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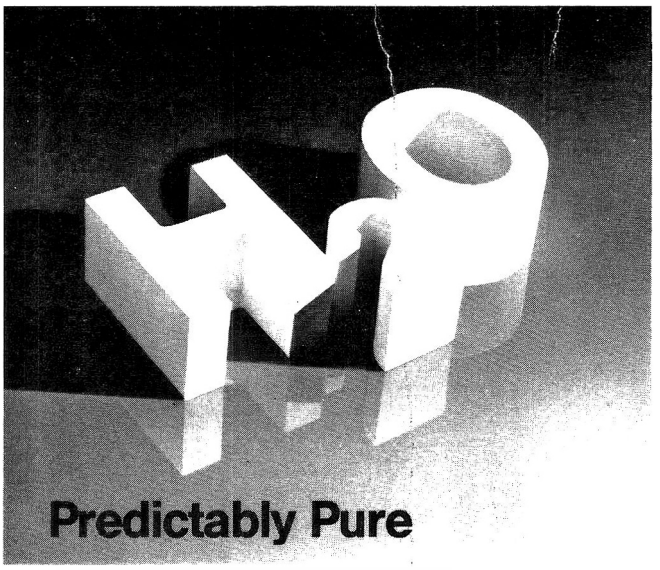

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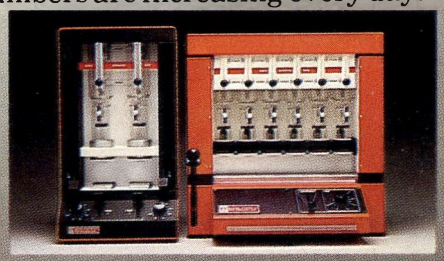

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Treatment and Disposal of Pesticide Wastes. Edited by R. E. Krueger and J. N. Seiber. Published by the American Chemical Society (ACS), 1155 16th St, NW, Washington, DC 20036, 1984. 373 pp. Price: $64.95 (U.S. & Canada)/$77.95 (export). ACS Symposium Series No. 259.

This publication outlines the problems of pesticide waste disposal by examining the chemical, biological, and physical methods of disposal, the basic principles underlying each of these methods, and the analytical and modeling approaches applicable to waste disposal conditions.

McGraw-Hill Dictionary of Chemistry. Published by McGraw-Hill Book Co., 1221 Avenue of the Americas, New York, NY 10020, 1984. 665 pp. Price: $32.50. ISBN 0-07-045420-5.

This edition contains more than 9000 entries concentrating on terminology of both theoretical and applied chemistry, and including biological compounds and industrial chemicals. Entries were selected from various disciplines of chemistry, including analytical chemistry.

Kirk-Othmer Concise Encyclopedia of Chemical Technology. Published by John Wiley & Sons, Inc., 605 Third Ave, New York, NY 10158, 1985. 1300 pp. Price: $129.95.

This third edition contains over 1100 updated articles from 1500 internationally recognized chemistry experts and has been reviewed for accuracy. This edition also includes illustrations, tables, charts, graphs, figures, and an indexing and cross referencing system.

Contemporary Practice of Chromatography. By C. F. Poole and S. A. Schuette. Published by Elsevier Science Publishers, PO Box 211, 1000 AE Amsterdam, The Netherlands, 1984. 708 pp. Price: $61.25 (U.S. & Canada)/Dfl. 159.00 (rest of world). ISBN 0-444-42410-5.

This book covers all areas of gas, liquid, and thin layer chromatography. Emphasis is on the practice of chromatographic methods, including "how to" sections and numerous examples of calculation methods.

Seafood Toxins. Edited by E. P. Ragelis. Published by the American Chemical Society (ACS), 1155 16th St, NW, Washington, DC 20036, 1984. 472 pp. Price: $79.95 (U.S. & Canada)/$95.95 (export). ACS Symposium Series No. 262.

This book, a collection of information on seafood toxic organisms that affect fish and shellfish, examines the impact of these toxins on seafood consumption, and discusses their chemistry, origin, geographical distribution, pharmacological action, and methods of detection.

Pharmaceutical Statistics. Practical and Clinical Applications. By S. Bolton. Published by Marcel Dekker, Inc., 270

Madison Ave, New York, NY 10016, 1984. 544 pp. Price: $75.00 (U.S. & Canada)/$90.00 (rest of world). ISBN 0-8247-7218-0.

This volume provides a thorough introduction to pharmaceutical statistics. Topics range from data graphics and probability to advanced applications, including factorial designs, quality control, and optimization techniques.

Analysis of Foods and Beverages. Modern Techniques. Edited by G. Charalambous. Published by Academic Press, Inc., Orlando, FL 32887, 1984. 672 pp. Price: $82.00. ISBN 0-12-169160-8.

This book presents the latest developments in not only chromatographic techniques but also in several newer ones—scanning electron microscopy, X-ray microanalysis, atomic spectrometry, bioassay, nuclear magnetic resonance, and others.

Chemical Ionization Mass Spectrometry. By A. G. Harrison. Published by CRC Press, Inc., 2000 Corporate Blvd, NW. Boca Raton, FL 33431, 1983. 168 pp. Price: $60.00 (U.S.)/$69.00 (rest of world). ISBN 0-8493-5616-4.

This book provides a review of the principles, instrumentation, and applications of chemical ionization mass spectrometry. It discusses chemical ionization systems that are widely used in analytical chemistry.

Determination and Assessment of Pesticide Exposure. Edited by M. Siewierski. Published by Elsevier Science Publishers, PO Box 211, 1000 AE Amsterdam, The Netherlands, 1984. 222 pp. Price: $75.00 (U.S. & Canada)/Dfl. 195.00 (rest of world). ISBN 0-444-42416-4.

This book includes papers that focus on the techniques and analytical methodology in monitoring human exposure to pesticides, the interpretation and use of exposure data, and descriptions of methods for reducing pesticide exposure.

The Agrochemicals Handbook. Published by the Royal Society of Chemistry. Distributed by Heyden & Son, Inc., 247 S 41st St, Philadelphia, PA 19104. 1984. 1000 pp. Price: £90.00/$171.00 (U.S.) ISBN 0-85186-406-6.

This handbook contains comprehensive data and information on a variety of substances that are active components of agricultural chemical products used in crop protection and pest control, including herbicides, insecticides, fungicides, nematicides, acaricides, and rodenticides.

Therapeutic Drug Monitoring and Toxicology by Liquid Chromatography. Edited by S. H. Y. Wong. Published by Marcel Dekker, Inc., 270 Madison Ave, New York, NY 10016, 1985. 520 pp. Price: $89.75 (U.S. & Canada)/$107.50 or SFr. 268.–(rest of world). ISBN 0-8247-7246-6.

This reference presents an overview of the fundamentals of therapeutic drug monitoring, recent clinical instrumentation topics, clinical pharmacology, and LC analysis of drugs.

Lange's Handbook of Chemistry. 13th Ed. Edited by J. D. Dean. Published by McGraw-Hill Book Co., 1221 Avenue of the Americas, New York, NY 10020, 1985. 1792 pp. Price $57.00. ISBN 0-07-016192-5.

This 13th edition contains new information including an expanded table of the pK_a values of 2300 organic acids; a revised table of the properties of combustible mixtures; material for current thermodynamic values for 3600 inorganic compounds; and capsule explanations, which describe such procedures as use of statistics, separation techniques, spectroscopic techniques, and thermodynamic relations.

Microcolumn Separations. Edited by M. V. Novotny and D. Ishii. Published by Elsevier Science Publishers, PO Box 211, 1000 AE Amsterdam, The Netherlands, 1985. 336 pp. Price: $64.75 (U.S. & Canada)/Dfl. 175.00 (rest of world). ISBN 0-444-42429-6.

This book, written by internationally recognized experts in microcolumn technology and miniaturized spectroscopic and electrochemical detection, summarizes advances in microcolumn liquid chromatography, capillary supercritical fluid chromatography, and microelectrophoresis.

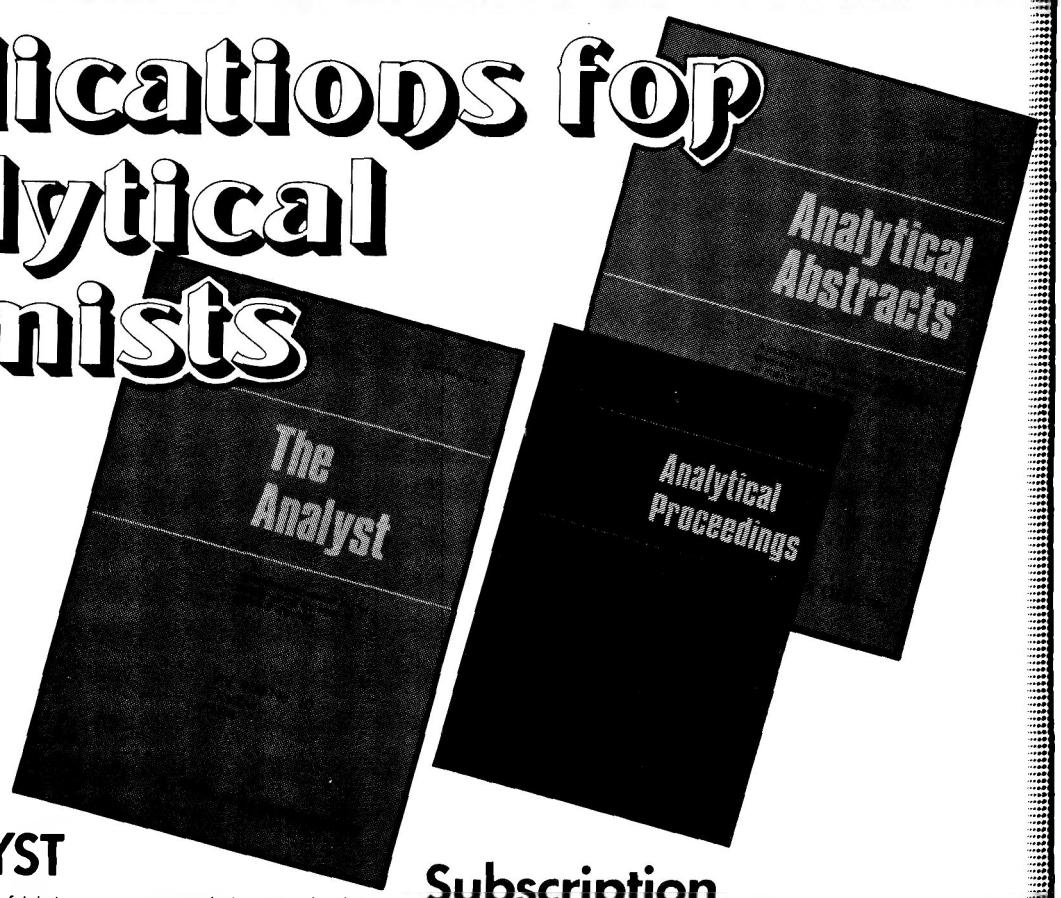
Advances in Cereal Science and Technology, Volume VII. Edited by Y. Pomeranz. Published by the American Association of Cereal Chemists (AACC), 3340 Pilot Knob Rd, St. Paul, MN 55121, 1985. 362 pp. Price: $49.00 (AACC members)/$60.00 (nonmembers). ISBN 0-913250-39-2.

This volume features 6 reports on important cereal science topics: seed storage proteins of economically important cereals, the phospholipases of cereals, changes in rice during parboiling, dietary fiber in cereals, sprouted grain, and starch damage.

Resonance Ionization Spectroscopy and Its Applications 1984. Edited by G. S. Hurst and M. G. Payne. Published by Adam Hilger. Distributed by Heyden & Son, Inc., 247 S 41st St, Philadelphia, PA 19104, 1984. 360 pp. Price: $49.00. Order No. 990380041.

This volume contains invited papers presented at the 2nd International Symposium on Resonance Ionization Spectroscopy and Its Applications, Knoxville, TN, in April 1984. Topics include introduction to the RIS technique, spectroscopy and kinetics, resonance ionization mass spectroscopy, analysis of solids, vacuum ultraviolet generation and noble gas detection, and possible applications of weak interactions in physics and particle physics.

Publications for Analytical Chemists



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CIRCLE 9 ON READER SERVICE CARD

EPA Project Summary

Eleven collaborating laboratories participated in an interlaboratory study for the analytical detection of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD) in water using the U.S. Environmental Protection Agency (USEPA) Method 613. 2,3,7,8-TCDD is labeled as a toxic "priority" pollutant under sec. 307(a) of the Clean Water Act Amendments of 1977.

In USEPA Method 613, water containing internal standard 2,3,7,8-TCDD is extracted with methylene chloride, concentrated, exchanged to hexane, and then subjected to capillary gas chromatography-mass spectrometric analysis, which allows for separation and measurement of the 2,3,7,8-TCDD isomer in the extract.

The interlaboratory study required analyses of 2,3,7,8-TCDD in 6 water types at each of 6 concentrations (3 Youden pairs) in addition to the analysis of all water blanks with no spiked compound. Each participating laboratory then forwarded a report to Monsanto Research Corp. containing all data obtained, and a completed questionnaire covering specifics on the analyses including sources of distilled, tap and surface waters; instrumentation used; GC conditions; and specific problems encountered in the analyses.

The final step in the study was a statistical analysis of all data by Battelle Columbus Laboratories, Columbus, OH, under USEPA Contract No. 69-03-2624 using the USEPA IMVS system of computer programs.

The objective of this study was to characterize the performance of Method 613 in terms of accuracy, overall precision, single-analyst precision, and the effects of water types on accuracy and precision.

Accuracy is obtained by comparing mean recovery to the true values of the concentration. Average percent recovery is 91% with a range from 86 to 96%.

The overall relative standard deviation (RSD) indicates the precision associated with measurements generated by a group of laboratories. Average percent relative standard deviation is 21% with a range of 18 to 23%.

The relative single-analyst standard deviation (RSD-SA) indicates the precision associated within a single laboratory relative to the mean recovery. Average percent relative standard deviation for a single analyst is 16% with a range of 13 to 18%.

A statistical comparison of the effect of the water types indicated no statistically significant differences between water types.

Correct activation of the alumina column is critical. Overactivation causes adsorption which results in incomplete or no elution of TCDD from the column.

Some laboratories experienced poor recovery and poor precision on the labeled compounds. As a quality control measure, a second independent internal standard or surrogate should be used to detect possible error in known concentration, evaporated concentration, or incomplete dissolution of standards.

Six of the 11 laboratories experienced little or no difficulty with Method 613. In these cases, over 98% of the data points were retained (4 outliers out of 216 analyses). All data from one laboratory which used an incorrect internal standard were rejected. Of the remaining 4 laboratories, 66% of the data were found to be outliers.

In conclusion, Method 613 is recommended for the analyses of 2,3,7,8-TCDD in municipal and industrial wastewaters.

CA Selects Bulletins

CA Selects is a series of bulletins published by Chemical Abstracts Service (CAS). Ten CA Selects bulletins fall under the analytical chemistry category: analytical electrochemistry, automated chemical analysis, chelating agents, forensic chemistry, inorganic analytical chemistry, organic analytical chemistry, surface analysis, thermal analysis, trace element analysis, and ultrafiltration. Fifteen others are grouped under chromatography or spectroscopy. These issues, which are published every 2 weeks, contain bibliographic information, abstracts of recent research and structure diagrams, and CAS Registry Numbers where applicable. Subscription price/year: $100.00 (CA subscribers), $110.00 (nonsubscribers). Residents of Europe and Japan should contact their CAS representatives and pay in local currencies. Contact: Chemical Abstracts Service, Customer Service Dept, 2540 Olentangy River Rd, PO Box 3012, Columbus, OH 43210, USA, 800/848-6538 (USA), 614/421-3600 (elsewhere).

Short Courses

The Association of Official Analytical Chemists (AOAC) will offer 2 short courses, one immediately before and one after the AOAC Annual International Meeting: Quality Assurance for Analytical Laboratories, October 26–27, 1985, and Microbiology Quality Assurance, October 31–November 1, 1985. Both courses will be held at the Shoreham Hotel, Washington, DC. To register, contact: Margaret Ridgell, AOAC, 1111 N 19th St., Suite 210, Arlington, VA 22209, USA, 703/522-3032.

The American Association of Cereal Chemists will offer 4 short courses: Computers in the Lab, Fall 1985, Chicago, IL; Nutrition, September 20–21, 1985, Orlando, FL; Gum Chemistry and Technology in the Food, November 13–15, 1985, Chicago, IL; and Starch: Structure, Properties, and Food Uses, December 5–6, 1985, Chicago, IL. For more information, contact R. J. Tarleton, AACC Headquarters, 3340 Pilot Knob Rd, St. Paul, MN 55121, USA, 612/454-7250.

The American Chemical Society (ACS) will offer 3 lecture/lab short courses: Microprocessors and Minicomputers: Interfacing and Applications, September 15–20, 1985, and December 15–20, 1985, Price: $695.00 (member), $765.00 (nonmember); Gas Chromatography: Packed and Capillary Columns, September 23–27, 1985, Price: $775.00 (member), $855.00 (nonmember); and Liquid Chromatography: Theory and Practice, December 10–13, 1985, Price: $695.00 (member), $775.00 (nonmember).

All courses will be held at Virginia Polytechnic Institute and State University, Blacksburg, VA. For information, contact Karen McIlvaine, Education Div., ACS, 1155 16th St, NW, Washington, DC 20036, USA, 202/872-4508.

Meetings

July 21–26, 1985: The American Association for Clinical Chemistry (AACC) 37th National Meeting, Georgia World Congress Center, Atlanta, GA. Contact the AACC Meetings Dept, 1725 K St, NW, Suite 1010, Washington, DC 20006, USA, 800/892-1400 or 202/857-0717.

August 14–15, 1985: Technicon Instruments Corporation's Industrial Systems Div. 8th International Symposium on Near Infrared Reflectance Analysis, Technicon Science Center, Tarrytown, NY. Contact: Mary Testani, Technicon Industrial Systems, 511 Benedict Ave, Tarrytown, NY 10591-5097, USA, 914/681-3083.

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HIGHLIGHTS

SYMPOSIA

Safety Training of Laboratory Analysts and Managers—Chairman: Robert Bianchi

Quality Assurance—Chairman: Susan G. Anderson

Immunotoxicity of Pesticides and Other Toxic Compounds: *Its Mechanisms and Up-to-date Methodology for Evaluation*—Chairman: Thomas S. S. Mao

Chromatography of Amino Acids and Determination of Amino Acid Composition of Nutritionally Important Protein Sources—Chairman: Charles W. Gehrke

Nutritional Analyses—Chairman: Robert C. Benedict

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Contributed papers on analytical subjects accepted for poster presentation.

For further information, contact Margaret Ridgell
AOAC, 1111 N. 19th St., Suite 210, Arlington, VA 22209 • (703) 522-3032

Circle 36 on Reader Service Card

September 3–6, 1985: Second International Symposium on the Synthesis and Applications of Isotopically Labeled Compounds, Vista International Hotel, Kansas City, MO. Contact: Donald Wilk, 5100 Rockhill Rd, Kansas City, MO 64110, USA, 816/276-1616.

September 29–October 4, 1985: Federation of Analytical Chemistry and Spectroscopy Societies meeting, Philadelphia, PA. Contact: Matthew S. Klee, Smith, Kline & French Laboratories, 709 Swedeland Rd, L25, Swedeland, PA 19479, USA, 215/278-7661.

October 1–3, 1985: Labcon Central '85: Laboratory Instrument and Equipment Conference and Exhibition, Chicago, IL. For more information on exhibits, contact: Tower Conference Management Co., 331 W Wesley St, Wheaton, IL 60187, USA, 312/668-8100.

October 7–9, 1985: The Department of Food Science and Nutrition will sponsor an international conference, Biotechnology in the Food Processing Industry, Minneapolis, MN. Contact: Lynette Marten, University of Minnesota, Office of Special Programs, 405 Coffey Hall, 1420 Eckles Ave, St. Paul, MN 55108, USA, 612/373-0725.

October 27–31, 1985: AOAC 99th Annual International Meeting and Exposition, Shoreham Hotel, Washington, DC. Contact: Margaret Ridgell, AOAC, 1111 N 19th St, Suite 210, Arlington, VA 22209, USA, 703/522-3032.

November 10–13, 1985: Society of Environmental Toxicology and Chemistry (SETAC) 6th Annual Meeting, Marriott Pavilion Hotel, St. Louis, MO. Contact: SETAC, PO Box 4352, Rockville, MD 20850, USA, 301/468-6704.

November 19–22, 1985: 24th Annual Eastern Analytical Symposium and Exhibition sponsored by the American Chemical Society and the Society for Applied Spectroscopy, New York Penta Hotel, New York City, NY. Contact: G. M. Nakane, EAS Printing and Direct Mail Chairman, PO Box 4000, Princeton, NJ 08540, USA, 201/846-1582.

April 27–30, 1986: AOAC 11th Annual Spring Training Workshop, Madison Hotel, Seattle, WA. Contact: H. Michael Wehr, Oregon Dept of Agriculture, Laboratory Services Div., 635 Capital St, NE Salem, OR 97310, USA, 503/378-3793.

June 1986: AOAC Midwest Regional Section Meeting, Lincoln, NE. Contact: Thomas Jensen, Nebraska Dept of Agriculture, 3703 S 14th St, Lincoln, NE 68502, USA, 402/471-2176.

September 15–18, 1986: AOAC 100th Annual International Meeting and Exposition, The Registry Resort and The Sheraton Scottsdale Resort, Scottsdale, AZ. Contact: Margaret Ridgell, AOAC 1111 N 19th St, Suite 210, Arlington, VA 22209, USA, 703/522-3032.

New Sustaining Members

AOAC welcomes the following new sustaining members to the growing list of organizations aware of the need to support an independent methods validation association: Perdue, Inc., Salis-

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For more information, contact M. Ridgell at above address or at 703-522-3032, (Cutside U.S.: + 1-703-522-3032)

(Photocopy this form for additional advance registrations.)

bury, MD; McLaughlin Gormley King Co., Minneapolis, MN; Kansas State Board of Agriculture, Division of Laboratories, Topeka, KS; Loma Linda Foods, Inc., Riverside, CA; and Beatrice/Hunt Wesson Foods, Inc., Fullerton, CA.

ISO Standards Published

The following standards have been published by the International Organization for Standardization (ISO), Technical Committee 34—Agricultural Food Products. The standards are available, at prices indicated, from American National Standards Institute, Inc., 1430 Broadway, New York, NY 10018; 212/354-3300.

ISO 8157-1984 Fertilizers and soil conditioners—Vocabulary—$24.00

ISO 6638-1984 Fruit and vegetable products—Determination of formic acid content—Part 2: Routine method—$12.00

ISO 7386-1984 Aniseed (*Pimpinella anisum* Linnaeus)—Specification—$12.00

ISO 6656-1984 Animal and vegetable fats and oils—Determination of polyethylene-type polymers—$12.00.

Standard Reference Materials

The National Bureau of Standards (NBS) Office of Standard Reference Materials announces the availability of the following Standard Reference Materials (SRM): SRMs 1871–1875, glasses for microchemical analysis, feature rod form (approximately 2 × 2 × 20 mm). Different matrices and dopants make these glasses useful in the analysis of complex unknowns. Price: $196.00 per set.

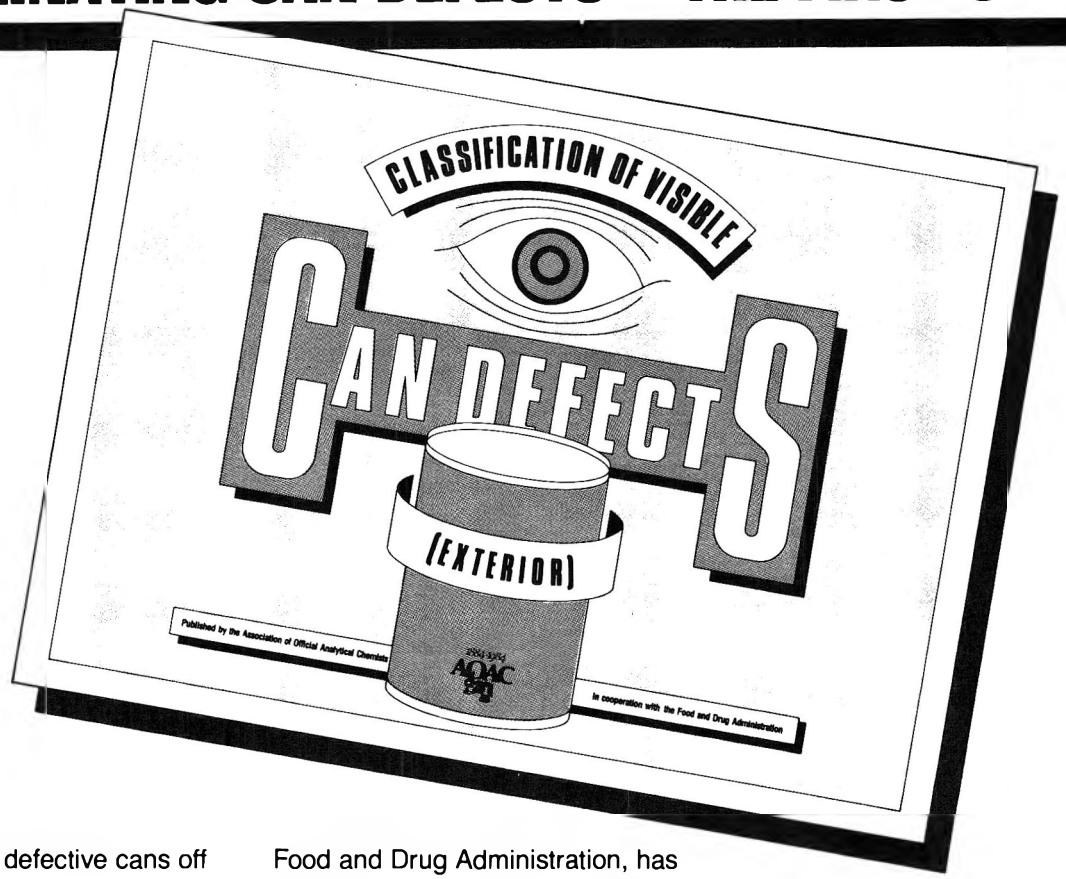
SRMs 185f, 187c, 188, 189, 186Ic and 186IIc, 191a and 192a, 922 and 923 are furnished as crystals for the preparation of buffer solutions of known hydrogen ion activity for calibration and checking the performance of commercially available pH materials and measuring systems. These SRMs are certified for pH measurements ranging from 3 to 10. Note: The last 6 SRMs listed are certified as admixtures only and should be purchased in pairs. Prices range from $63.00 to $75.00.

These SRMs may be purchased from: Office of Standard Reference Materials, B311 Chemistry Bldg, National Bureau of Standards, Gaithersburg, MD 20899; 301/921-2045.

Interim Method

The following method has been accepted interim official first action by the Methods Committee on Microbiology and Chairman of the Official Methods Board: Enumeration of Total Bacteria and Coliforms in Milk by Dry Rehydratable Media Film, submitted by R. E. Ginn (Dairy Quality Control Institute), V. S. Packard (University of Minnesota), and T. L. Fox (Riker Laboratories). The method will be submitted for adoption as official first action at the 99th Annual International Meeting of AOAC, Oct. 27–31, 1985, at Washington, DC. Copies of the method are available from the AOAC office.

ELIMINATING CAN DEFECTS—THE FIRST STEP



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The chart is a valuable reference resource for food processors, salvage operators, retail food personnel, wholesalers and state and local government sanitarians.

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The Association The primary objective of the Association of Official Analytical Chemists (AOAC) is to obtain, improve, develop, test, and adopt uniform, precise, and accurate methods for the 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 agriculture, public health, 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.

Membership Membership in AOAC is open to all interested persons worldwide. Sustaining memberships are available to any government agency or private company interested in supporting an independent methods validation program.

The Journal The Journal of the Association of Official Analytical Chemists is published by AOAC, 1111 N 19th St, Suite 210, Arlington, VA 22209. The Journal is issued six times a year in January, March, May, July, September, and November. Each volume will contain approximately 1400 pages. The scope of the Journal broadly encompasses the development and validation of analytical procedures pertaining to both the physical and biological sciences related to agriculture, public health and safety, consumer protection, and the quality of the environment. Emphasis is focused on research and development to test and adopt 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.

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INSTRUCTIONS TO AUTHORS

Scope of Articles

The Journal of the AOAC will publish articles that present, within the fields of interest of the Association *(a)* unpublished original research; *(b)* new methods; *(c)* further studies of previously published methods; *(d)* background work leading to development of methods; *(e)* compilations of authentic data; *(f)* technical communications, cautionary notes, and comments on techniques, apparatus, and reagents; *(g)* invited reviews of methodology in special fields. The scope broadly encompasses the development and validation of analytical procedures pertaining to both the physical and biological sciences related to agriculture, public health and safety, consumer protection, and quality of the environment. Emphasis is focused on research and development to test and adopt precise, accurate, and sensitive methods for the analysis of foods, food additives, supplements and contaminants, cosmetics, drugs, toxins, hazardous substances, pesticides, feeds, fertilizers, and the environment. Compilations of authentic data include monitoring data of pesticide, metal, and industrial chemical residues in food, tissues, and the environment. All articles are reviewed for scientific content and appropriateness.

Preparation of Manuscript

Authors must submit 3 copies of the complete manuscript, including tables and illustrations, to AOAC, 1111 N 19th St, Suite 210, Arlington, VA 22209. The manuscript is to be typewritten on one side of white bond paper, 8½ × 11 inches, with page margins of 1 inch, and **double-spaced** throughout (i.e., title, authors' names and addresses, footnotes, tables, references, captions for illustrations, and the text itself). Tables are to be typed on separate sheets, *not* interspersed through the manuscript. Drawings and photographs should be mounted apart from the text or submitted as separate items.

Style and Format

The text should be written in clear, concise, grammatical English. Unusual abbreviations should be employed as little as possible and must always be defined the first time they appear. Titles of articles should be specific and descriptive. Full first names, middle initial (if any), and last names of authors should be given. The address of the institution (including zip code) from which the paper is submitted should be given and should be in a form to which inquiries, proofs, and requests for reprints can be sent. Information supplementing the title and names and addresses should be given as footnotes.

Methods, Results and/or Discussion, Acknowledgments, and Recommendations (applicable to reports of General and Associate Referees) should be placed in sections under appropriate headings.

Abstracts: Each manuscript should be accompanied by a concise abstract (not more than 200 words). The abstract should provide specific information rather than generalized statements.

Introduction: Each article should include a statement on why the work was done, the previous work done, and the use of the compound being studied.

Methods: Methods should be written in imperative style, i.e., "Add 10 mL . . . Heat to boiling . . . Read in spectrophotometer." Special reagents and apparatus should be separated from the details of the procedure and placed in sections with appropriate headings; however, common reagents and apparatus or those which require no special preparation or assembly, need not be listed separately. Reagents and apparatus listed should be described in generic terms and in terms of performance; use of brand names should be avoided. Hazardous and/or carcinogenic chemicals should be noted. Any very long, detailed operation can be given in a separate section with an appropriate heading (e.g., Preparation of Sample; Extraction and Cleanup; Preparation of Standard Curve). Any necessary calculations should be included; number of significant figures must reflect the accuracy of the method. Wherever possible, metric units should be used for measurements or quantities.

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Illustrations: Illustrations, or figures, may be submitted as original drawings or photographs; photocopies are acceptable for the two review copies but not for the printer's copy. All figures must be cited in the text consecutively. Figures are numbered by arabic numbers, and all figures must be accompanied by descriptive captions, typed on one (or more) separate sheets, *not* on the figure itself. The figure should be identified by number on the back by a soft pencil or (preferably) a gummed label.

Drawings should be submitted either as the original drawing or a good glossy photograph; photocopies, multiliths, Verifax copies, Xerox copies, etc. are *not* acceptable. Drawings should be done in black India ink (ordinary blue or blue-black ink is not acceptable) or with drafting tape on white tracing paper or tracing cloth or on "fade-out" graph paper (ordinary graph paper ruled with green or dark blue ink is not acceptable). Lettering should be done with a Leroy lettering set, press-on lettering, or a similar device; freehand or typewritten lettering is not acceptable. Values for ordinate and abscissa should be given, with proper identification conforming to Journal style (example: wavelength, nm), at the sides and bottom of the figure. Lettering or numbering on the face of the figure itself should be kept at a minimum; supplementary information should be given in the caption. Several curves on the same figure should be identified by simple symbols, such as letters or numbers, and the proper identification or explanation given in the caption. Letters and numbers should be large enough to allow reduction to journal page or column size. The Journal does not publish straight line calibration curves; this information can be stated in the text.

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References: References to previously published work should be collected at the end of the article under the heading "References." Each item in the list is preceded by an arabic number in parentheses. Every reference must be cited somewhere in the text in numerical order (rather than alphabetical or chronological). It is the author's responsibility to verify all information given in the references.

References to journal articles must include the following information: last names and at least one initial of *all* authors (*not* just the senior author); year of publication, enclosed in parentheses; title of journal, abbreviated according to accepted *Chemical Abstracts* style; volume number; numbers of first and last pages. References to books, bulletins, pamphlets, etc. must include the following information: last names and initials of authors or editors; year of publication, enclosed in parentheses; full title of book; volume number or edition (unless it is the first edition); publisher; city of publication; numbers of pertinent pages, chapter, or section. Citation to private communications or unpublished data should be included in the text, *not* in the list of references.

Spectrophotometric, gas chromatographic, and liquid chromatographic nomenclature should follow the practice recommended by the American Society for Testing and Materials.

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FOCUS: INTERNATIONAL FERTILIZER METHODS

Influence of AOAC Fertilizer Analysis Methods Beyond North America

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The analytical methods used for regulatory purposes in each country are standardized or adopted by national standards organizations or concerned committees. These bodies either develop new methods or adopt already available analytical methods as such or with suitable modifications to meet their requirements. Analytical methods in common use in some countries, namely, the United Kingdom, Japan, Indonesia, Malaysia, Thailand, Pakistan, India, Brazil, Ecuador, Peru, and Colombia, as well as those adopted under European Economic Community (EEC) directives and International Organization for Standardization (ISO), have been reviewed to assess the influence of AOAC.

The determination of phosphorus (gravimetric) by the quinolinium phosphomolybdate method as standardized by AOAC has been accepted by Japan, Indonesia, and India. This method is also near final acceptance by ISO. Countries like the United Kingdom, France, The Netherlands, and Poland also favor this method. The spectrophotometric and volumetric determinations of phosphorus as standardized by AOAC are also used in many countries. The method used for determination of potash is Japan, India, Malaysia, and Indonesia is the AOAC STPB method (volumetric), while ISO and countries such as the United Kingdom prefer a gravimetric estimation of potash. Japan uses both methods. The Lindo-Gladding (gravimetric) method of estimating potassium, adopted by AOAC but relegated to surplus status, does not appear to be in much demand.

The conventional Kjeldahl method of determining nitrogen is still used throughout the world, with suitable modifications regarding types of catalysts. The Ulsch reduced iron method is being used in many EEC countries, Japan, and, to some extent, India. AOAC prefers Robertson's method and the Jones modification for estimation of nitrates (indirect) while ISO has adopted the nitrate estimation (gravimetric) involving the use of Nitron. The comprehensive nitrogen method of AOAC is popular for estimating all forms of nitrogen present in a sample. Both AOAC and ISO prefer the use of chromium as reducing agent. AOAC specifies Cr in its comprehensive method only. Other AOAC methods use Raney catalyst, Zn, or thiosulfate as reducing agents. Se is never used in AOAC methods, and the modified comprehensive method substitutes $CuSO_4.5H_2O$ for HgO.

For moisture estimation, many countries have accepted the Karl Fischer method as standardized by AOAC but without using dioxane. The gravimetric estimation of moisture, adopted by AOAC, is also widely used. AOAC methods of micronutrient estimations are applicable mostly for very low level micronutrients; for high analysis fertilizers, additional methods may have to be included. It is generally observed that conventional wet chemical analytical methods are preferred over instrumental methods for regulatory purposes where accuracy is more important than speed of analysis.

India specifies only AOAC methods for testing the quality of imported fertilizers from the United States, the Soviet Union, and many European and Middle East countries. For determination of physical parameters in fertilizers, India has adopted methods from the standards literature and from those developed by the Indian Standards Institute. Some Latin American countries like Colombia and Ecuador have specified in their regulations that only AOAC methods are to be used.

Because AOAC methods are being adopted in many countries, their influence is also carried to ISO through its member bodies as well as to EEC countries. With a long standing, sound procedure of testing analytical methods through worldwide collaborative studies, AOAC has been the leader in the field of fertilizer analysis during its first one hundred years.

In view of the recognized importance of fertilizers in agriculture, several countries had introduced legislative measures designed to control their production, trade, and distribution as early as the 19th century. For instance, the Fertiliser Law in France was enacted in 1888 and has continued, through suitable amendments, to meet the changing needs of the times (1). Due to growing realization by consumers of the importance of standard quality fertilizers, regulatory measures are presently in force in almost every country in the world. The various legislative provisions generally include definitions of relevant terms. The term "fertilizer" as is generally understood refers to the manufactured material or a substance which is inorganic or organic in nature and contains one or more of the essential elements required for plant growth. Fertilizer terminology distinguishes between nutrient elements and nutrients, and between nutrient materials and fertilizers. Fertilizers are either pure nutrient materials or nutrient materials mixed or combined with other substances that may or may not be useful to plants but are not harmful if suitably used. The term fertilizer has to be appropriately understood to apply the laws regulating that substance legally defined as fertilizer and also to select appropriate analytical methods for testing its quality.

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Definitions of Fertilizers

Various national laws have defined fertilizers specific to their requirements. However, some countries like Belgium, Egypt, Morocco, and Sri Lanka have adopted a list of fertil-

izer types in their legislations and have not thought it necessary to define the fertilizer. Japanese law defines fertilizer as all substances applied to the soil for the purpose of supplying plant nutrients or producing a chemical change in the soil to contribute to the cultivation of plants. According to the Indian Fertiliser (Control) Order (1957), fertilizer is any substance applied to the soil and specified in column 1 of Schedule I (2). At present, only NPK, and Zn-bearing materials are regulated. This definition is being modified to include sources of all essential nutrients, including liquid fertilizers and, if possible, microbial fertilizers. In The Netherlands, any product added to the soil to maintain or increase productive capacity is considered a fertilizer.

In New Zealand, animal manure and other animal or vegetable matter, either fresh or partly decomposed, are not considered fertilizers unless they have been treated in some way to assure that the composition is stable until the material is applied to the soil or plants. The Colombian law distinguishes fertilizers, soil amendments, and soil conditioners all of which are defined separately without an overall definition for the term fertilizer. The Federal Republic of Germany Fertiliser Act explicitly includes, under the definition of fertilizers, preparation of soil inoculates, soil conditioners, and plant growth regulators but without further defining them. The legislation in Switzerland also covers bacterial cultures, artificial amendments, and compost acceleration. In countries like Norway, the title of the law itself distinguishes fertilizers and soil amendments; both commodities are covered in the provisions. Peruvian legislation of fertilizers applies to guano for agricultural use. In Mexico, fertilizer is defined as any substance, either Mexican or imported, intended for use in its natural state or after processing for the purpose of soil fertilization (1).

These few examples suggest that, apart from conventional N, P, and K-bearing fertilizers, the soil amendments, organic materials, bio-fertilizers, and trace elements are also regulated through statutory controls and would, therefore, need appropriate methods of sampling and analysis.

Basis for Selecting a Method

A legislative act or order provides for the types of fertilizers that could be manufactured or imported for sale in a country. The specifications with regard to the minimum nutrient content, the maximum limits of permissible impurities and moisture, as well as the particle size distribution are laid down in the act or order issued to regulate the quality of fertilizers in a country. The collection of samples and analysis of fertilizers is carried out according to pre-determined procedures.

The preparation of a sample for analysis is an important step in the chain of activities in an analytical laboratory. Apart from the quality of method of analysis, the quality of sample handling and preparation is a crucial factor which influences the results of analysis. The scope of this paper is, however, confined to the analytical methods; the sampling aspect is to be dealt with separately.

The attributes of an analytical method should be its accuracy, precision, specificity, sensitivity, applicability, practicability, speed, and cost (3). With advancements in analytical techniques and the advent of computer-controlled instrumental methods of analysis, it is possible to make simultaneous multinutrient analyses on a sample.

An analytical method is selected depending on the purpose of analysis. In process control, the method chosen may be simple, relatively accurate, but fast; for quality control/enforcement purposes, the accuracy, precision, sensitivity, and reproducibility of the results are more important considerations.

The adoption of uniform methods of analysis for judging the quality of fertilizers, by the seller, the buyer, and the regulator, becomes very crucial. If methods of analysis are not uniform, the results obtained by the laboratories cannot be expected to be uniform. Not only do laboratories within a country have to follow uniform methods, it is becoming necessary that laboratories throughout the world follow the same method. For import or export of fertilizers, 2 countries have to agree on the same method of analysis to obtain comparable results; this also helps the international tribunals settle disputes related to composition and quality of fertilizers.

Agencies Developing and Adopting Analytical Methods

The Association of Official Analytical Chemists since its formation 100 years ago has taken up the task of selecting methods of analysis from published literature or developing new methods, and then, before adoption, testing the methods through collaborative, international, interlaboratory studies for a variety of materials relating to food, drugs, agriculture, and products affecting public health and welfare. The collaborative studies are published in the AOAC *Journal*, and the official adopted methods are collected and published in the compendium *Official Methods of Analysis* of the AOAC.

This system greatly serves the need of government regulatory bodies for analytical methods. Due partly to the long history of use, but mostly to the scientifically sound system of screening and accepting or developing and adopting a method, AOAC methods are in use throughout most of the world. AOAC methods are also used by researchers and other scientists, but their primary applicability is for regulatory purposes.

Each country, however, has to adopt a method or a set of methods for a particular determination according to requirements of the legislation. In the Federal Republic of Germany, for instance, the method of analysis is prescribed by the Organisation of German Agricultural Testing and Research Institute. In Belgium, analyses are carried out in accordance with methods approved by the Minister of Agriculture; in the absence of such methods, or if they cannot be applied, the analysts follow traditional methods in use in analytical laboratories and government stations. In Switzerland, the Agricultural Testing Stations of Lausanne and Liebefeld Berne are responsible for enforcement and prescribe the method to be used for analysis. Similarly, in Kenya, methods as described in Fertiliser and Animal Feed Stuffs (Analysis Rules)(1970) are used. In Malawi, methods prescribed in 6th Schedule of Fertiliser Regulations (1970) are followed. The Fertiliser (Sampling and Analysis) Amendment Regulations (1980) specify the methods of analysis being followed in the United Kingdom. For those fertilizers that are covered by EEC directives, EEC methods of analysis are used. The International Organization for Standardization or the individual National Standards Institutions, like British Standards Institution in England and Indian Standards Institution in India, publish standard test methods for fertilizers. Many of these standards-setting organizations use AOAC methods.

This paper reviews the existing statutory methods for fertilizer analysis being followed in EEC countries, by the International Organization for Standardization (ISO), and by the United Kingdom, Japan, Indonesia, Malaysia, Pakistan, Thailand, Brazil, Ecuador, Peru, Colombia, and India. It is not customary in some of the countries to cite the reference of the method adopted from a particular source. Inferences have been drawn from the description of a method and by

comparison with the AOAC procedures. AOAC numbers for relevant methods have been given as reference (4).

Fertilizer Analytical Methods in Different Countries

EEC and ISO

The member states of the European Economic Community (EEC) are under the obligation to abide by the specifications and testing procedures laid down in EEC directives for fertilizers which are called "EEC-type fertilizers." EEC harmonization is meant to facilitate free movement, trade, and inspection of fertilizers in accordance with predetermined and commonly agreed specifications and testing methods. The application of EEC methods is obligatory in arbitration cases. However, in daily work, an EEC member country can use simpler and labor-saving methods. EEC members have the responsibility to ensure that fertilizers bearing the name "EEC-type fertilizers" or fertilizers of EEC standards are subjected to frequent official inspections with regard to guaranteed nutrient content, forms of nutrient, and nutrient solubility. It is also the responsibility of member states to ensure that fertilizers which bear EEC standards and which are produced in a third country are subjected to the same official inspection as are those produced in a member state. EEC members are also members of ISO through their respective national standard bodies; thus, there is an interaction between ISO and EEC. AOAC methods, having acceptability among a large number of countries, also influence ISO methods through various members of ISO, including the American National Standards Institute (ANSI). It may be mentioned that EEC directives apply only to fertilizers of common interest to member states; for other fertilizers, the analytical methods of respective countries, or AOAC, or ISO are adopted.

For total nitrogen estimation, when nitrate is present, many EEC countries use the Ulsch or the Arnd method for reduction of nitrate to ammonia. ISO (5315) and AOAC recommend the use of chromium powder for reduction in acid solution (**2.059**). ISO has also standardized a gravimetric method using Nitron (ISO 4176) for determination of nitrate nitrogen as a referee method.

For phosphate estimation, the following extractants have been approved in accordance with EEC directives (5): extraction by mineral acid; extraction by 2% citric acid; extraction by 2% formic acid; extraction by neutral ammonium citrate; extraction by alkaline ammonium citrate at 65°C (Petermann); extraction by alkaline ammonium citrate at ambient temperature (Petermann); extraction by alkaline ammonium citrate according to Joulee; extraction by water.

Although the identification of a suitable extractant is of crucial importance so far as the crop requirement of a particular form of phosphorus is concerned, the fertilizer laboratory has to use an extractant which is predetermined through soil-test crop correlation studies carried out in the field, and is not directly involved in evaluating the suitability of an extractant. In EEC directives, phosphorus is determined by the quinolinium phosphomolybdate (gravimetric) method with the precipitating reagent free from acetone. While the United Kingdom has adopted the EEC method by legislation it has, nevertheless, supported the use of acetone in the method being considered by ISO.

A comparative study made by ISO (6) reveals that both methods (with and without the use of acetone) give identical results (Table 1).

The representative bodies in ISO from France, The Netherlands, Poland, the United Kingdom, and India have suggested the use of acetone in the method, which is likely to become an ISO standard method for determination of phosphorus. AOAC has been recommending the use of acetone for years (**2.026**). With the formal adoption of this method by ISO, there will be harmonization between ISO and AOAC for phosphorus determination. Acceptance of this method by United Kingdom, France, and a few more countries should promote acceptance by EEC as well. Then a long-standing difference in approach will be reconciled between AOAC and ISO as well as a few other countries.

A similar situation is the standard EDTA titration method which was modified by our Institute to determine zinc in zinc sulfate in the presence of magnesium by masking Mg with the use of formaldehyde–acetic acid solution. Magnesium is a common adulterant in $ZnSO_4$ fertilizers. The method involves the use of cyanide. Others feel that the estimation of zinc as ZnS (gravimetric) or estimation by a colorimetric determination (**2.184, 2.185**), although cumbersome, may be preferred because it does not use cyanide. In such a situation, it becomes difficult to reconcile the views of the 2 approaches.

For potash determination, ISO has accepted the potassium tetraphenylborate gravimetric method as a reference method (ISO 5318). AOAC has, however, been advocating the volumetric estimation by STPB (**2.119**). AOAC also recommends the use of platinum hexachloride for gravimetric estimation of K (**2.094**).

United Kingdom

A few of the methods embodied in the UK Fertiliser (Sampling and Analysis) Regulations (7) have been compared with AOAC methods.

Total nitrogen in urea is determined by simple acid digestion (involving no catalyst) followed by traditional alkali distillation. Nitrogen is determined in the presence of nitrate by Ulsch's reduced iron method. Nitrate nitrogen is determined by reduction as ammonia with the traditional Devarda's alloy. The AOAC method uses salicylic acid and thiosulfate for nitrate estimation (**2.058**). In comprehensive nitrogen methods of AOAC (**2.059, 2.061**), chromium is also used along with thiosulfate. Biuret is determined spectrophotometrically in a similar manner to the AOAC method (**2.088**).

Ammoniacal nitrogen is determined in the presence of urea by distillation with K_2CO_3. In the view of fertilizer companies

Table 1. International interlaboratory tests (ISO/DIS-6598) of precipitating reagent used in quinolinium phosphomolybdate method

	Solution A (10.438 mg P_2O_5)		Solution B (12.397 mg P_2O_5)	
Statistic	With acetone	No acetone	With acetone	No acetone
Arith. mean of results, 24 labs	10.413	10.410	12.381	12.397
SD of repeatability	0.022	0.030	0.017	0.0148
SD of reproducibility	0.065	0.047	0.080	0.0641
Diff. between mean and real value	−0.025	−0.028	−0.014	−0.0164

all over the world who manufacture DAP with at least 2% nitrogen as urea and 16% ammoniacal nitrogen, it is important that a suitable method is studied and adopted by AOAC. The methods suggested by Viswanathan et al. (8) for determination of ammoniacal nitrogen in the presence of urea deserve consideration.

The gravimetric estimation of potassium is carried out by precipitating K in a slightly alkaline medium in the form of potassium tetraphenylborate involving the use of STPB. In contrast, AOAC (**2.119**) prefers volumetric estimation of potassium by using excess sodium tetraphenylboron for precipitation of K and titrating excess STPB with benzalkonium chloride, using clayton yellow as indicator. In UK legislation, the perchloric acid method and flame photometric method (**2.108**) for K have also been adopted.

Asian and Far East Countries

In accordance with the decision taken in a seminar on fertilizer legislation (9), conducted by FFTC in Japan in 1971, it was agreed by the participants to prepare a model of fertilizer legislation designed to accommodate the needs of developing countries within Southeast Asia regions and to describe in detail the model requirements of legislation covering the purpose, definition, official standards, registration, manufacture, guarantee labels, inspection, and analysis. It was suggested that the analytical techniques should be uniform for all the Asian countries. This decision strengthened the universally recognized need and importance of following uniform methods of analysis. A study of the prevailing analytical methods in a few countries in Asia and the Far East, namely, Japan, Indonesia, Malaysia, Thailand, Pakistan, and India, shows a dominating influence of AOAC methods in these countries. A brief description is given below.

Japan.—In accordance with official methods published by the National Institute of Agricultural Sciences, Ministry of Agriculture and Forestry, Tokyo (10)(1977), moisture in fertilizers like single superphosphate (SSP), triple superphosphate (TSP), ammonium sulfate, sodium nitrate, and muriate of potash is determined gravimetrically by an oven-drying method as described by AOAC (**2.013**). Moisture in urea is determed by heating the samples at 75°C; the AOAC method involving the use of Karl Fischer titrant (**2.016**) is preferred in most other countries.

Total nitrogen is estimated in nitrate-containing fertilizers by using salicylic acid, similar to the AOAC method (**2.058**). In nitrate-free fertilizers, total nitrogen estimation by the sulfuric acid method is different from AOAC (**2.058**) with regard to the use of catalyst. Instead of expensive and toxic HgO, the more conventional $CuSO_4$ is used as a catalyst in Japan. Total nitrogen in nitrate-containing fertilizers having only water-soluble forms of nitrogen is determined according to the Ulsch reduced iron method. This method is recommended in the presence of chlorides. The alkali distillation of ammoniacal nitrogen and the formaldehyde titration for estimation of ammonical nitrogen is similar to the AOAC (**2.066**) method. Nitrate nitrogen is estimated according to the Devarda's alloy method. The phenol-disulfonic acid method as well as a UV spectrophotometric method are also followed in Japan. Urea nitrogen is determined by enzymatic hydrolysis of urea. This is also an AOAC accepted method (**2.080**).

For determination of phosphorus, the gravimetric and volumetric quinoline-molybdophosphate methods as well as spectrophotometric vanadium phosphomolybdate methods are recommended, which are similar to AOAC methods (**2.026, 2.029,** and **2.021,** respectively).

Potash estimation is carried out both volumetrically as well as gravimetrically with the aid of sodium tetraphenylboron. AOAC does not provide for gravimetric estimation of K. In Japan, potash is also determined by emission spectrophotometry and absorption spectrophotometry.

Zinc is estimated by spectrophotometry as well as by atomic absorption spectrophotometry. Both methods are also official in AOAC (**2.187, 2.186**).

Indonesia.—Indonesia is one of the few countries where a comprehensive body for fertilizer legislation did not exist until recent years. From the time of Dutch rule until 1956, all fertilizers were analyzed by the Institute of Chemical Industries, as well as several other organizations. No inspection system existed in this country although the Superintending Company of Indonesia Ltd, local manufacturing units, customs officials, brokers, and consumers were taking samples for analysis. Uniform sampling procedures and analytical methods obviously did not exist. There was no legal basis for penalizing dealers who sold adulterated or mislabeled fertilizers. According to the study carried out by the National Fertiliser Development Center, TVA, for the Agency for International Development, various testing laboratories in Indonesia claimed that they use AOAC methods for testing fertilizers. However, systematic adoption of any particular method was not done until 1970.

A report, "Fertilizer Quality Control in Indonesia" (11), prepared on the request of AID/Indonesia in September 1973, detailed model regulations, definitions of appropriate terms, registration, labeling, inspection, sampling, and analysis. The methods described for analysis of total content and various forms of nitrogen, phosphorus, and potassium were those of AOAC (1970). In some cases, the Fertilizer Institute's (TFI) analytical methods have also been suggested for adoption in Indonesia.

Malaysia.—The Malaysian Standards are prepared by the Technical Committee on Fertilisers under the authority of the Material and Chemical Industry Standards Committee. The methods are periodically reviewed to keep abreast of progress in the area concerned. In most cases, official AOAC methods are endorsed for adoption as Malaysian Standards for analysis of fertilizers (12). In some cases, the methods in Fertilisers and Feeding Stuff Regulations of Great Britain have also been adopted. A perusal of individual methods reveals that moisture in fertilizers is estimated by oven-drying which is similar to AOAC (**2.013**). Total nitrogen estimation (nitrate-free samples) is done by a standard Kjeldahl method with HgO as catalyst (**2.055**); ammoniacal nitrogen is determined by NaOH distillation (**2.065**); total, water-soluble, and citric acid-soluble P_2O_5 are determined volumetrically as well as colorimetrically in accordance with AOAC methods (**2.119, 2.122**).

The estimation of ammoniacal nitrogen in the presence of urea nitrogen is carried out by distillation under reduced pressure. Nitrate-containing samples are analyzed by using Devarda's alloy, while urea nitrogen is estimated by the urease method (**2.068, 2.080**). These analyses are carried out in the same sample by first converting urea nitrogen to ammoniacal nitrogen, followed by reduction of nitrate nitrogen to ammoniacal nitrogen and then combined distillation.

The chloroplatinate (gravimetric) and flame photometric methods are used for K estimation, which are similar to AOAC methods (**2.095** and **2.108**). The colorimetric estimation of zinc (dithizone) and the use of atomic absorption spectrophotometry follow AOAC methods (**2.185, 2.186**).

Thailand.—In Thailand, fertilizer manufacture, import, and trade is regulated in accordance with Act BE 2518, enforced

since 1975 (13). The Act lays down the specifications of fertilizers, tolerance limits. registration, inspection procedures, sampling, and analysis. For the determination of nitrogen and phosphorus, AOAC methods are followed; potash is determined by the official methods of analysis of fertilizers of the National Institute of Agricultural Sciences, Ministry of Agriculture and Forestry, Japan (1977), which provide for estimation by both flame photometry and atomic absorption spectrophometry. Variations in analysis obtained between the 2 standard laboratories in Thailand are reported to be 1% each for nitrogen and phosphorus and 2% for K_2O.

Pakistan.—The Fertiliser Standards in Pakistan are adopted by the Pakistan Standards Institue on the recommendation of the Fertiliser and Allied Products Selection Committee (14). A typical study of Pakistan standards 217:1975 (UDC:633:814.7) for urea shows that moisture estimation is carried out gravimetrically by drying fertilizers 4 h at 75°C. The Karl Fischer method has also been recommended for estimation of moisture in urea. However, instead of extracting moisture with dioxane and then titrating with Karl Fischer reagent, as recommended in AOAC (**2.016**), methyl alcohol is used. Total nitrogen is analyzed by the Kjeldahl method, with copper sulfate as catalyst instead of HgO as recommended in AOAC (**2.055**). Biuret estimation is carried out spectrophotometrically by measuring the color at 555 nm which is developed with copper under alkaline conditions. Tartrate is used to prevent precipitation of copper as copper hydroxide. The method is the same as AOAC (**2.088**).

Latin American Countries

A brief reference to relevant legislation and analytical methods in Brazil, Colombia, Peru, and Ecuador is made by FAO (15). In Brazil, the legislation regulates the trade in fertilizers and soil amendments. In Colombia, the purpose is to control domestic trade, while in Ecuador, the laws are applicable to fertilizer quality and soil amendments. The Peruvian laws are applicable to guano.

Brazil.—In Brazil, the trade in fertilizer is regulated in accordance with Decree No. 8169 (November 6, 1948) and subsequent relevant changes. The Agricultural Chemical Institute of the National Centre for Agronomical Research and a few other official laboratories are authorized to carry out chemical analysis of fertilizers. Plant nutrients must be expressed in percentage of nitrogen, phosphorus (P_2O_5), potassium (K_2O), and calcium (CaO). The forms of phosphorus must be expressed as 2% citric acid-soluble, ammonium citrate-soluble, and insoluble forms. Nitrogen must be expressed as organic, nitrate, or ammoniacal nitrogen. AOAC methods are among the methods adopted for analysis of fertilizers in Brazil.

Colombia.—In Colombia, Decree No. 1057 (November 20, 1953) on trade in fertilizers provides for regulations which are similar to those of Brazil. With regard to the guarantee of fertilizer specifications, the total nitrogen as well as the proportions of organic, nitric, and ammoniacal nitrogen have to be specified. The available phosphoric acid and water-soluble potash have to be specified. Under Article 10 of the Decree, it has been specified that the analytical methods to be applied are those of the AOAC.

Ecuador.—The quality and sale of fertilizers is regulated through legal decree. Fertilizers intended for sale are required to bear a label with the name and address of the manufacturer/importer or vendor as well as the composition of the product expressed as follows: total nitrogen; nitrogen soluble in water (ammoniacal and nitrate nitrogen); total P_2O_5; phosphoric acid soluble in ammonium citrate; potash soluble in water, expressed as potassium oxide; total lime, expressed as calcium oxide; natural phosphates (phosphate rock and thomas slag) with obligatory indication of the degree of fineness or pulverization.

The AOAC methods of analysis are adopted in Ecuador.

Peru.—Peruvian legislation on fertilizers stipulates that all activities associated with the official control and sale of guano are the responsibility of the Guano Administration Corporation Organisation (Decree No. 3069, January 1919). In accordance with Decree 13 of the soils regulations, the Kjeldahl method is to be followed for analysis of nitrogen content and the Pemberton method for determination of phosphoric acid in guano. The Central Control Laboratory of the Corporation may modify and adopt methods from other sources.

India

In Indian agriculture and, thus, in Indian economy, fertilizer plays a very important role. According to the FAO indicative world plan (1), the projected figures for consumption of nutrients in developing countries for the period 1985–86 is 33 million tons. The share for India is expected to be about 10 million tons. India is the second largest importer of fertilizers in the world. India imports fertilizers from the United States, Canada, East and West Germany, The Netherlands, Czechoslovakia, Bulgaria, Yugoslavia, the Soviet Union, Abu Dhabi, Kuwait, Qatar, Italy, and Jordan. India, therefore, interacts with a large number of countries in the world in fertilizer trade and occupies an important position in terms of the extent of this activity as well as the amount of fertilizer imported, produced, and consumed. It is, therefore, necessary that India adopts analytical methods for checking quality of fertilizers which are both acceptable in the exporting countries and are scientifically sound and capable of testing fertilizers within acceptable limits of analytical variations. India has preferred that AOAC methods be used to test fertilizers before they are shipped to India, and this system has been acceptable to the various exporting agencies. On arrival at Indian ports, imported fertilizers are again tested with AOAC methods prescribed for the purpose.

In a country the size of India, where fertilizer has to be made available to millions of farmers, most of whom are illiterate and have very small and marginal farm holdings, it was recognized that effective regulation on fertilizer production, distribution, pricing, and quality alone can ensure timely availability in the required quantity and right quality. With this objective, fertilizer was declared an essential commodity (March 1957) under the provisions of Section 2 of the Essential Commodities Act (ECA) (1955).

The specifications indicate the guaranteed (minimum) content of total nutrients in various fertilizers and their forms. The (maximum) permissible limits of impurities, moisture content, free acidity, biuret (wherever applicable), and particle size distribution in fertilizers have been prescribed. The following types of analysis are required to be carried out (2): Nitrogen: total nitrogen in nitrate-free samples; total nitrogen in nitrate-containing samples; total nitrogen in fertilizer with high $Cl:NO_3$ ratio, like nitrophosphates; ammoniacal nitrogen; nitrate nitrogen; combined ammoniacal and nitrate nitrogen; water-insoluble nitrogen; urea nitrogen. Phosphorus: total; water-soluble; citrate-soluble; citrate-insoluble; and 2% citric acid-soluble. Potassium: water-soluble form is estimated by perchloric acid and STPB (titrimetric) methods in water extract. Miscellaneous estimations: various miscellaneous estimations required to judge quality include zinc in zinc sulfate, moisture, free acidity, arsenic, free phosphoric

acid, calcium nitrate, chlorides, copper, lead, and magnesium.

Procedure Followed for Adoption of Methods in Fertiliser (Control) Order.—A review of methods included in the Fertiliser (Control) Order (1957) shows that most are AOAC methods. A few methods from Fertiliser (Sampling and Analysis) Regulations of the United Kingdom have also been accepted, as well as by the Indian Standards Institution, particularly for estimation of physical parameters. Adopting, updating, and revising is done by the Ministry of Agriculture through the advice of an Expert Committee representing the Indian Council of Agricultural Research, Indian Agricultural Research Institute, agricultural universities, the fertilizer industry, and the Central Fertiliser Quality Control and Training Institute, Faridabad. Methods are circulated among the members for their comments. The method may be referred to the Central Institute for examination and comments. Considerations are accuracy, repeatability of results, speed of analysis, and availability of chemicals. In general, most methods adopted are wet chemical analyses. AOAC methods which are designed preferably as reference methods for regulatory purposes are considered suitable for adoption. Instrumental methods of analysis can be better used by the fertilizer manufacturers for "on-line analysis" where speed of analysis is given primary importance. In instrumental methods, the cost and maintenance of equipment become limiting factors for large scale adoption.

The methods adopted in the Fertiliser (Control) Order (1957) are uniformally followed by 43 statutory fertilizer quality control laboratories in different states in India, including the Central Fertiliser Quality Control and Training Insitute. The Central Laboratory also functions as a referee laboratory. The capacity of the fertilizer control laboratories is estimated at 70 000 samples per year, while the fertilizers are sold through a network of wholesalers and retailers numbering over 1 450 000. Because of this gap between analysis requirements and available analytical facilities, quick tests are needed for screening the prima facie substandard/adulterated samples at the inspection stage itself. These preliminary tests help inspectors to forward suspected samples on priority to the laboratories for detailed testing by the statutory methods. Motsara and Singh had developed a quick qualitative testing kit (16, 17). The initial reaction from the field about the utility of the kit has been favorable. Large-scale use of the kit is being further promoted by training the inspecting staff on its use and handling.

In many developing countries, a gap exists between required analytical capacity and available facilities. AOAC may consider developing qualitative tests and devices like a "quick testing kit" for screening tests in the field.

Inter- and Intralaboratory Check System in India.—Along the lines of the Magruder Check Sample System organized in the United States, the Central Institute at Faridabad has introduced a check sample system to monitor the analytical skill and accuracy of fertilizer control laboratories in India. According to this system, fertilizer samples of known (confidential) composition are periodically sent to all the laboratories. The analysis is carried out by the methods prescribed in the Fertiliser (Control) Order. The performance of the laboratories is evaluated on the basis of their reports. This system helps to identify the problems, if any, with laboratories performing the correct analysis. The system of "reverse check" has also been started. According to this system, the Central Institute randomly obtains (every 100th) a portion of the already analyzed sample from various fertilizer control laboratories for testing at the Central Institute. Simultaneously, the results from laboratories are also obtained. By camparing the results, it is judged whether every laboratory is applying skill and accuracy in performing fertilizer analysis on a day-to-day basis. The Central Institute proposes to associate itself with the Magruder Check Sample System so that the analytical accuracy and skill of this Institute is assessed through an international program.

A User's View of AOAC Methods

AOAC has served a great purpose, through supplying widely acceptable analytical methods for a range of commodities, to the regulating authorities in many parts of the world. Still more demands exist for AOAC's consideration on more fruitful utilization of existing information on methods and on development of more methods for further use.

(1) AOAC methods are considered to be too brief; also many back and forth references are made in a method to other methods. As a result, the Fertilizer Institute Product Quality Committee in the United States has produced a *Manual on Fertilizer Sampling and Analysis* which is considered to be a very useful laboratory manual (18). In India, Motsara and Tripathi (19) produced *A Laboratory Handbook for Fertiliser Analysts* wherein all methods included in the Fertiliser (Control) Order have been described in detail with the principle involved and calculation required in each estimation. The description of each method is complete. In view of the fact that the AOAC book of methods covers a wide range of products, the volume of the book would increase enormously if further method details were given. However, the acceptability of AOAC methods and their use throughout the world may be important considerations for publishing lengthier and more complete descriptions for convenience of users. Perhaps AOAC methods for various commodities should be supplied in separate volumes. Pesticides and other related areas could also form part of a fertilizer volume.

(2) Methods for estimating some physical qualities of fertilizers are not given in AOAC, for example, determination of particle size, bulk density, particle hardness, angle of repose, etc.

(3) Some countries have unconventional fertilizers, like salica in Japan and guano in some Latin American countries, which require the availability of acceptable analytical methods.

(4) Methods for estimation of minor constituents and impurities present in fertilizers, like $Ca(NO_3)_2$, free phosphoric acid, free acidity, and arsenic, are not given by AOAC.

(5) Methods for estimation of micronutrients are not given in as great detail as those for major elements.

(6) The applicability and limitations of each method are not always given.

(7) In view of the increasing cost of fertilizers, the use of bacterial fertilizers like *Rhizobium* and *Azatobecter* cultures is receiving more and more attention in both developed and developing countries. In India, the preparation of *Rhizobium* culture may be brought under statutory control for regulating quality since the product is already appearing on the market. Some countries, like Switzerland, already cover bacterial cultures under their statutes for regulating fertilizers. The AOAC may consider standardizing test methods for this group of fertilizers. Also, analytical methods for future chemical fertilizers like polyphosphates and micronutrient-fortified fertilizers may be considered.

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SPICES AND OTHER CONDIMENTS

Liquid Chromatographic Determination of Protein Acid Hydrolysate Content in Blended Soy Sauce

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Five methods were investigated for the determination of levulinic acid in soy sauce to determine the addition of protein hydrolysate, mainly acid hydrolysate of defatted soybeans. Best results were obtained by using liquid chromatography (LC) with 0.004M $HClO_4$ as the mobile phase and bromcresol purple as a post-column reagent. An innovative LC method with 0.1% H_3PO_4 as eluant was developed for determination of levulinic acid at 280 nm in soy sauce. This was the most time-saving method.

Protein in soybeans is broken down mainly to amino acids through either fermentation or acid hydrolysis to produce soy sauce. Fermented soy sauce (FSS) is superior in odor and taste to acid hydrolysate of defatted soybeans (AHDS), but the maximum degree of protein hydrolysis by fermentation is much less than that by acid hydrolysis; moreover, fermentation takes much longer. Therefore, it is economically profitable to adulterate FSS with AHDS in commercial soy sauce products. This study was initiated to develop a rapid and accurate method for determination of AHDS in blended soy sauce (BSS), and to compare it with methods in the literature.

Acid hydrolysis of protein destroys all the tryptophan and part of the tyrosine, methionine, cystine, and serine, etc. (1). Fermented soy sauce contains some organic acids, for example, succinic acid, which were not found in AHDS (2). However, those differences could not serve as indices for discrimination between FSS and AHDS because amino acids and organic acids might be added artificially to soy sauce.

Levulinic acid in AHDS originates from acid action on carbohydrates in defatted soybeans (1, 3). Under conditions usually employed, i.e., 6N HCl, 110°C, and 24 h, the content of levulinic acid varies from 900 to 1100 mg/g total nitrogen in AHDS (1), depending on the level of carbohydrates in the defatted soybeans. Levulinic acid is very stable (4); it is the main source of unpleasant acid flavor in AHDS soy sauce (5). Ion exchange resin (6) or microorganisms (7) were reported to remove part of the levulinic acid from soy sauce, but their use is economically impractical. FSS does not contain levulinic acid, and the latter has never been an additive in FSS. Therefore, levulinic acid is a proper index for determination of AHDS content in BSS.

Qualitative tests that use levulinic acid to discriminate FSS and AHDS include a vanillin–H_2SO_4 method (8), and thin layer chromatography (2). Quantitative assays to determine AHDS in BSS include the vanillin–H_2SO_4 method (9); spectrophotometric measurement of the reaction of levulinic acid and hydrazine at 242 nm (10, 11); gas chromatography (GC) (8, 12); silica gel liquid chromatography (13); 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride colorimetric reaction (14); and liquid chromatography (LC) (15).

We investigated the usefulness of 5 rapid methods for determining AHDS in BSS: UV spectroscopy (10, 11), GC (12, 16), isotacophoresis (IP) (17), and 2 LC methods (15). The first 3 procedures are detailed in the respective reports. The 2 LC methods are described here.

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Experimental

Soy Sauce Samples

Pure fermented and chemical soy sauces were provided by 5 main soy sauce manufacturers in the Taiwan area, A, B, C, D, and E. Pure fermented soy sauce of another manufacturer (K) was used in the recovery test.

Apparatus

(a) *Liquid chromatograph.*—Model 204 (Water Associates, Milford, MA 01757), equipped with Model M-6000 psi pump, Model M-45 4000 psi pump, Model U6K loop injector, Model 440 UV detector with sensitivity range 0.005–2.0 absorbance unit full scale (AUFS) and 280 nm wavelength filter, Model 480 variable UV detector with sensitivity range 0.002–2.0 AUFS, column temperature controller, and Model 730 recording integrator.

(b) *Silica cartridge.*—Sep-Pak (Waters Associates).

(c) *Cation exchange LC column.*—8 mm × 250 mm, 10–20 μm sulfonate type porous styrene-divinyl-benzene copolymer (Shodex c-811, Showa Denko K. K., Minato-ku, Tokyo, Japan). Two columns were connected and placed in temperature-controlled box.

(d) *Cation exchange LC precolumn.*—8 mm × 50 mm, 20–30 μm, same packing material described in (c).

(e) *Filter.*—Type GSWP, 0.22 μm pore size (Millipore Corp., Bedford, MA 01730).

Reagents

(a) *Levulinic acid.*—(Sigma Chemical Co., St. Louis, MO 63118).

(b) *Indicator reagent.*—For LC at 430 nm: Dissolve 108 mg bromcresol purple and 8.9535 g $Na_2HPO_4 \cdot 12H_2O$ in deionized water, add 2 mL 20% $HClO_4$, and dilute to 1 L with deionized water to make 2 × 10^{-4}M bromcresol purple in 0.25M Na_2HPO_4 and 0.004M $HClO_4$ buffer.

(c) *Mobile phases.*—For LC at 430 nm: 0.004M $HClO_4$. For LC at 280 nm: 0.1% H_3PO_4.

Sample Preparation

Soy sauce was diluted by adding an equal volume of deionized water, and was filtered through membrane filter (0.22 μm).

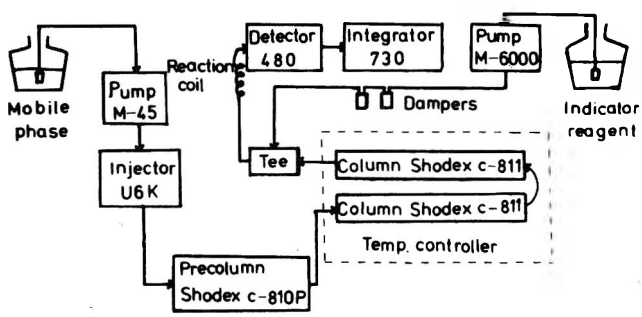

Figure 1. Diagram of post-column detection system for LC at 430 nm.

Table 1. Recovery of levulinic acid added to soy sauce,[a] measured by LC with detection of 2 wavelengths

Authentic concn,[b] mg/mL	Measured concn,[c] mg/mL		Recovery, %	
	430 nm	280 nm	480 nm	280 nm
0	0	0		
2.51	2.59	2.54	103	101
5.02	4.95	5.40	99	108
10.03	10.01	9.62	100	96
15.03	15.40	14.17	102	94

[a]Mean of 2 or 3 measurements.
[b]A mixture of FSS of K mfr with standard solution to desired concentration of levulinic acid, mixing ratio: v/v = 1/1.
[c]Calculated by external standard method, using integrator.

Table 2. pH and absorbance at 430 nm of low concentrations of levulinic acid in mixtures (v/v = 1/2) of indicator reagent and mobile phase

Concn,[a] ppm		pH[b]	Absorbance[c]
0		6.815	0.000
3		6.817	0.003
60		6.746	0.051
100		6.673	0.087
300		6.326	0.334
	Indicator reagent	7.486	
	Mobile phase	2.545	
	Deionized water	6.144	

[a]Mix by volume 1 part of indicator reagent with 1 part mobile phase, and dissolve accurately weighed amount of levulinic acid in the mixture to desired concentration.
[b]Measured by pH meter.
[c]Measured by JASCO spectrophotometer (D_2 lamp).

Determination

(a) *LC at 430 nm.*—Figure 1 shows the LC post-column detection system assembled in the laboratory. Levulinic acid and 8 other main organic acids in soy sauce were measured at 430 nm. Chromatographic conditions were as follows: flow rate of mobile phase 1.0 mL/min, flow rate of indicator reagent 0.5 mL/min, sensitivity range 0.2, chart speed 0.5 cm/min, column temperature 38°C, and sample size 10 μL. Mobile phase and indicator reagent were filtered (0.22 μm) before use.

(b) *LC at 280 nm.*—Sample was injected into the LC system under the following chromatographic conditions: flow rate of mobile phase 1.2 mL/min, column temperature 64°C, sensitivity range 0.05, chart speed 1.0 cm/min, and sample size 10 μL.

Results and Discussion

Recovery Test

Recovery of levulinic acid added to FSS was measured by the 5 methods. Good recovery was obtained with the LC methods, but interferences were found when the UV, IP, and GC methods were used. However, FSS from sources A, B, and C contained apparent levulinic acid when analyzed by methods other than LC. LC and GC peaks were identified by retention time.

(a) *LC at 430 nm.*—Only 4 reports concerning LC analysis of organic acids in soy sauce were found in the literature (18–21). The LC method used in this study was provided by the Central Research Laboratory of Kikkoman Co. (Noda, Japan). It is a modification of the method in Shodex technical data

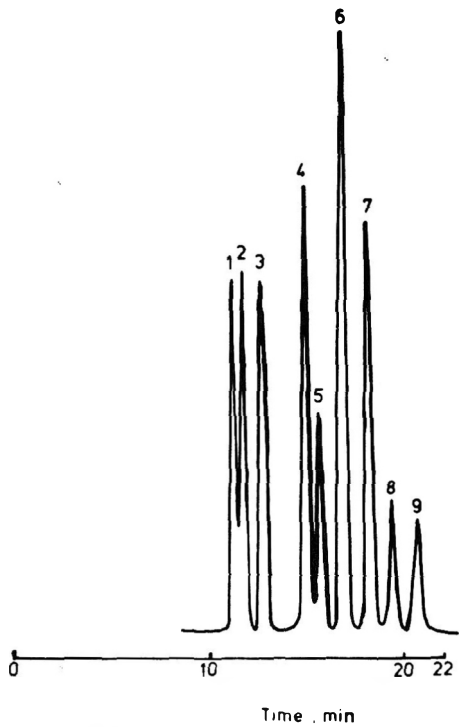

Figure 2. Chromatogram of organic acids standard mixture at 430 nm: 1, citric acid; 2, tartaric acid; 3, malic acid; 4, succinic acid; 5, lactic acid; 6, formic acid; 7, acetic acid; 8, levulinic acid; 9, pyroglutamic acid.

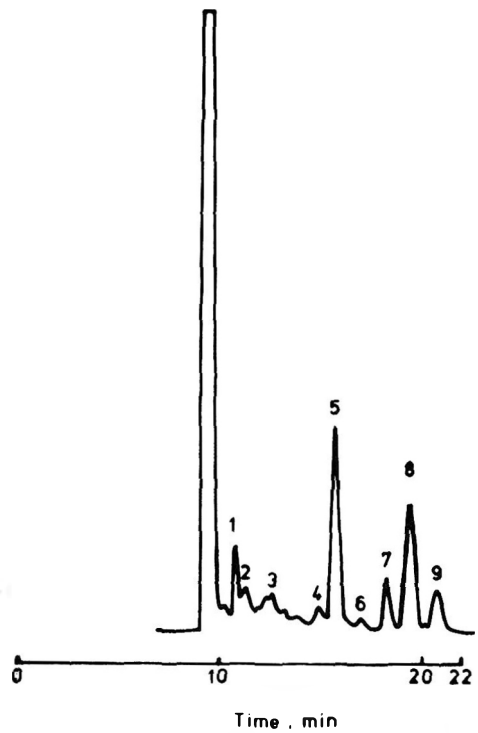

Figure 3. Chromatogram of organic acids in soy sauce at 430 nm: 1, citric acid; 2, tartaric acid; 3, malic acid; 4, succinic acid; 5, lactic acid; 6, formic acid; 7, acetic acid; 8, levulinic acid; 9, pyroglutamic acid.

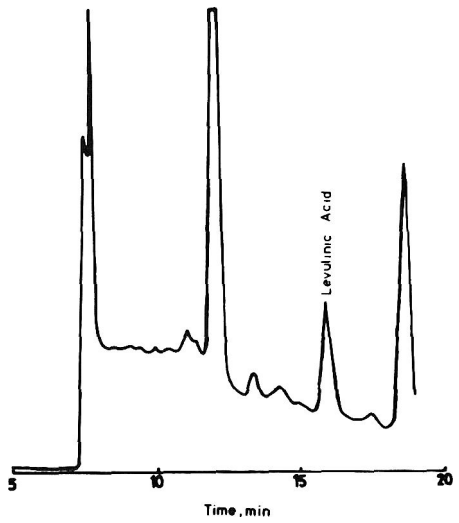

Figure 4. Chromatogram of levulinic acid in soy sauce at 280 nm.

Table 3. Total nitrogen (TN) of AHDS and FSS from 5 soy sauce manufacturers

	TN,[a] w/v %	
Mfr	AHDS	FSS
A	2.12	1.62
B	1.98	1.52
C	2.03	1.57
D	1.91	1.57
E	2.51	1.61

[a]Mean of 2 or 3 measurements.

(15), and is based on the same principle described in part 3 of Yoshida's report (20). Recovery tests showed that LC with detection at 430 nm was the best among the 5 methods tested (Table 1). Nine main organic acids were separated and measured in 22 min (Figures 2, 3).

(b) *LC at 280 nm.*—Recovery for the LC method (Table 1) was much better than that for the UV, GC, and IP methods. At 17 min per analysis (Figure 4), it was also the most rapid of the 5 methods tested. Compared to LC at 430 nm, instrumentation was simpler because no indicator reagent was used. LC analysis of organic acids has used 200–220 nm wavelength to determine the $-COOH$ group, or used a refractive index detector for measurement (18, 19, 22). In our study, analysis at 280 nm for the $-CO$ group of levulinic acid eliminated interference from most other organic acids.

Lowest Detection Limit by LC at 430 nm

Table 2 shows the absorbance at 430 nm of various levels of levulinic acid in a mixture of indicator reagent and mobile phase. According to Hamilton's "Calculations of Analytical Chemistry" (23), pKa_2 of phosphoric acid is 6.7 at 25°C. The calculated pH of mobile phase mixed with indicator reagent (v/v = 2/1) was 6.68; Table 2 gives the measured pH value of the mixture as 6.815. Bromcresol purple is yellow at pH <5.2 and purple at pH >6.8 (24). Acids were eluted from the column at pH = 2.5 as HA; their ionic forms A^- were detected by post-column reaction with bromcresol purple at pH 6.7. Buffering capacity of phosphate buffer was the greatest at pH = pKa. Therefore, at pH 6.7, the amount of acid required for a pH value change, i.e., for absorbance at 430 nm to change, was the least. Consequently, the method could detect a significant amount of acid but was not sensitive enough for small quantities of organic acids. The lowest practical detection limit for LC at 430 nm was 100 ppm levulinic acid.

Determination of AHDS in BSS

The degree of hydrolysis of soybean protein by microbiological fermentation has an upper limit, usually 57%, while acid hydrolysis achieves 100% hydrolysis of protein (1). Therefore, total nitrogen (TN) of soy protein hydrolysate is always higher than that of FSS, and was used as a co-index to determine AHDS in BSS (12). To calculate the amount of AHDS in BSS, the following formula was used:

$$\%\ \text{v/v, AHDS in BSS} = [(\text{mg/mL levulinic acid in BSS})/(\%(\text{w/v})\ \text{TN in BSS/F})] \times 100\%$$

where F = (mg/mL levulinic acid in AHDS)/(% (w/v) TN in AHDS).

General factor (F_G) in the LC at 430 nm method was about average of all the F values of the 5 soy sauce manufacturers. For the other methods, higher than average values were chosen in order to correspond in the 40–60% mixing range of BSS. F_G values of LC at 430 nm and LC at 280 nm were 11 and 14, respectively.

Total nitrogen of AHDS and FSS for the 5 soy sauce manufacturers is shown in Table 3. Tables 4 and 5 show determination of AHDS content in BSS. Best results were obtained using LC at 430 nm.

The small amount of levulinic acid detected by LC at 430 nm in fermented soy sauces from manufacturers D and E (Table 4) probably came from either inferior quality caramel additive (25), or contamination of residual AHDS from the storage tank.

In addition to the level of carbohydrates in raw material, many factors affect levulinic acid content and thus the F value

Table 4. Determination of AHDS in BSS, measured by LC at 430 nm

Authentic concn of AHDS in BSS,[a]	Concn of levulinic acid,[b] mg/mL					Measured concn of AHDS in BSS, v/v %				
v/v%	A	B	C	D	E[c]	A	B	C	D	E[c]
100	21.19	19.26	23.36	17.97	28.85	91	88	105	86	104
80	16.98	15.57	17.42	14.60	22.09	76	75	82	72	86
60	11.98	10.97	12.76	11.63	16.26	56	56	63	59	69
40	7.73	7.60	8.19	8.72	11.41	39	41	42	46	53
20	4.25	3.96	4.77	5.35	6.45	22	22	26	30	33
0	0	0	0	2.44	1.22	0	0	0	14	7

[a]Mixtures prepared in the laboratory using AHDS and BSS products from each manufacturer as ingredients.
[b]Mean of 2 or 3 measurements.
[c]Soy sauce manufacturers.

Table 5. Determination of AHDS in BSS, measured by LC at 280 nm

Authentic concn of AHDS in BSS,[a] v/v%	Concn of Levulinic acid,[b] mg/mL					Measured concn of AHDS in BSS, v/v %				
	A	B	C	D	E[c]	A	B	C	D	E[c]
100	23.32	26.21	24.81	20.34	32.32	78	95	87	76	117
80	17.84	20.66	21.86	17.15	26.04	63	78	80	66	102
60	14.13	15.94	14.20	13.46	19.80	53	64	55	54	84
40	9.80	11.19	10.51	9.96	13.80	38	47	43	42	64
20	4.72	5.87	5.37	6.20	8.07	20	26	23	27	41
0	0	0	0	3.00	1.06	0	0	0	14	6

[a–c]See footnotes in Table 4.

of AHDS. During acid hydrolysis of soybeans at about 100°C, levulinic acid content increases during the first 10 h and maintains constant thereafter. High temperature favors both the degree of hydrolysis and the formation of undesirable by-products, such as humus (reaction products of amino acids and furfural), levulinic acid, and formic acid. A low concentration of HCl plus a high ratio (w/w) of HCl solution vs soybeans favors levulinic acid synthesis but depresses the formation of humus (1). The manufacturers hydrolyze soybeans under compromise conditions to obtain the highest degree of hydrolysis, least consumption of energy and HCl, and least formation of humus.

Calculated from Tables 3 and 4, F values of 5 soy sauces range from 9.4 to 11.5, indicating that AHDS was manufactured under similar conditions. Therefore, accurate estimation of AHDS is applicable to commercial blended products using an F_G value of 11 for LC at 430 nm. Results for laboratory-prepared blended soy sauce, using AHDS and FSS ingredient products from the 5 sources (Table 4), and of a survey of 10 commercial soy sauces (unpublished data) show that LC at 430 nm is the most accurate method for estimation of AHDS adulteration of FSS.

Figure 4 and Table 5 show that LC at 280 nm is also a rapid and accurate method and requires less complicated equipment and reagents. Therefore, LC at 280 nm is more suitable for routine analysis of AHDS in BSS among the 5 methods tested.

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Volatile Oil Analysis of Cassia Bark (Cinnamon): Investigation of Systematic Errors

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Results of these studies demonstrate that steam-distillable volatile oil within a lot of cassia bark is heterogeneously distributed; that sample preparation can contribute a significant part of the overall analytical error; and that the latter error can be reduced by specifying that the sample be ground to pass a U.S. 20 mesh screen rather than a U.S. 40 mesh screen. An *F*-distribution value of 6.7 was calculated for the data, from 6 laboratories, used for statistical analysis. The critical *F*-distribution is 5.05, suggesting a significant difference in the means of the U.S. 20 mesh data and the U.S. 40 mesh data. The problem of sampling segregated materials where the functional form of the quality distribution is not known is briefly discussed.

The technique currently used to determine the amount of steam-distillable volatile oil in spices originated with the work done by Clevenger (1) in 1928. A number of studies have been made on the method and many improvements have resulted (2–8). These efforts culminated in approval of the method by the American Spice Trade Association (ASTA) (9) in 1968.

No comprehensive statistical studies were made of collaborative data on steam-distillable volatile oil of spices until the work sponsored by ASTA was completed by the Midwest Research Institute (MRI) (10) in 1965. However, it is interesting that the standard deviations reported in some early work were of the same order of magnitude as those reported in the MRI studies.

Little, if any, attention has been paid to the method of sampling cassia bark or to the details of preparing samples for analysis. The major concern seems to be that the samples be stored under refrigeration to prevent loss of volatile oil. The MRI report dismissed the question of the origin of samples with the statement that they were furnished by the Chairman of the ASTA Research Committee. It is clear that the objective of the work was to improve the analytical method, not to study sample preparation.

Procurement practice is to specify that cassia bark meet a minimum requirement of volatile oil content; this practice increasingly points out the disagreements between laboratory analyses of a given lot of cassia bark.

Preliminary work in the author's laboratory indicated that one of the significant variances is the large differences in the amount of volatile oil in the subsamples removed from a given lot of imported cassia bark. In light of questions raised by the spice trade industry as the result of these observations and others, the Executive Committee of the Technical Group of ASTA appointed a subcommittee to study 3 issues that could contribute to the variances observed by laboratories analyzing samples drawn from the same lots of cassia bark: the magnitude of subsample variances; the effect of variations in sample preparation technique; and the effect of the screen mesh.

Experimental

Study 1

Each of 6 firms who routinely run volatile oil analysis on cassia bark were asked to take a random sample consisting of 10 subsamples from a single lot of cassia bark in their inventories and run a volatile oil analysis in duplicate on each subsample. They reported all data, not just averages. ASTA method 16.0 (11) was used for the analysis. The results are shown in Table 1.

It is evident from the 1-way analysis of variance (Table 2) that large differences exist in the volatile oil content of the respective subsamples: The sum of squares (SS) values of the between-subsample data are large compared with the SS values of the duplicates. *F*-Valucs indicate a significant difference in the volatile oil content at the 95% confidence level between subsamples for a given laboratory.

Study 2

The second study was designed to reveal the degree of variance contributed by sample preparation, including kind of mill used, amount of analytical sample passed through the selected screen, and disposition of the oversized particles.

Two lots of thoroughly blended cassia bark were prepared at 2 locations. Approximately 1 lb subsamples were removed from these lots, coded, and sent to each of 9 participating in-house laboratories. The laboratories were asked to run one analysis only on each sample, using ASTA method 16.0. They were asked to report the analytical results and the following information: (a) kind of sample mill used to prepare the sample for analysis (i.e., brand, model no., or description of grinding principle (cutting, pulverizing, impact, etc.); (b) amount of subsample passed through the mill; (c) mesh size of screen used either in the sample mill or after grinding, or both, if the mesh size used in the final screening was different from that used in the mill. The results and statistical analysis are reported in Table 3.

Communication with laboratory 8 revealed that they used a cutting mill for sample preparation which had knives that had not been sharpened for an extended period. The calculated r value of the Dixon test for outliers was 0.60. The rejection limit is $r = 0.48$ (12). The data set from laboratory 8 was considered an outlier and was not used in the statistical analysis.

The *F*-ratio of the data was calculated by using an approach presented by Youden and Steiner (13). The results support the hypothesis that sample preparation does contribute to the overall analytical error.

Study 3

Because of comments from the participating laboratories regarding the grinding of samples to pass a U.S. 40 mesh screen, a study to explore the difference in reported volatile oil content of a well blended sample of cassia bark ground to pass a U.S. 20 mesh screen and one to pass a U.S. 40 mesh screen was established.

One 450 g subsample from a large, thoroughly blended lot of cassia bark was sent to each of 9 participating laboratories. They were requested to grind one portion of the subsample in such a fashion that at least 99% of the cassia bark passed through a U.S. 20 mesh screen; the remaining portion of the subsample was ground to pass 99% through a U.S. 40 mesh screen. The same mill was to be used in grinding each of the sample aliquots. The oversized particles were to be discarded.

The participants were cautioned to be sure their mills were in good working order. The ground, screened samples were

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Table 1. Study 1: Data and statistical analysis for volatile oil of cassia bark (mL/100 g)

Dupl.	1	2	3	4	5	6	7	8	9	10
					Subsample					
					Laboratory 1					
A	2.97	2.63	3.26	2.54	2.66	2.51	2.94	2.60	2.71	2.29[a]
B	3.09	2.86	3.11	2.80	2.63	2.66	2.97	2.63	2.63	2.60
			SS between subsamples			0.7645				
			SS within analysis			0.0945				
			F-ratio calculated			8.09				
			F-ratio critical (5% level)			3.18				
					Laboratory 2					
A	1.28	1.31	1.14	1.20	1.14	1.26	1.37	1.25	1.34	1.23
B	1.23	1.31	1.23	1.23	1.17	1.14	1.26	1.29	1.29	1.17
			SS between subsamples			0.0626				
			SS within analysis			0.0233				
			F-ratio calculated			2.99				
			F-ratio critical (5% level)			3.02				
					Laboratory 3					
A	1.82	2.03	2.34	1.91	2.32	1.91	2.03	1.94	1.63	1.80
B	1.75	2.06	2.20	1.91	2.37	2.05	2.19	2.14	1.66	1.75
			SS between subsamples			0.876				
			SS within analysis			0.058				
			F-ratio calculated			16.71				
			F-ratio critical (5% level)			3.02				
					Laboratory 4					
A	1.89	2.05	2.17	2.17	2.31	1.89	2 03	2.31	1.89	1.89
B	1.89	1.89	1.89	2.17	2.42	1.74	2 03	2.31	1.89	2.03
			SS between subsamples			0.605				
			SS within analysis			0.079				
			F-ratio calculated			8.50				
			F-ratio critical (5% level)			3.02				
					Laboratory 5					
A	2.60	3.00	1.86	2.49	2.86	2.29	2.43	2.57	2.54	2.14
B	2.57	2.89	1.94	2.43	2.86	2.23	2.49	2.66	2.60	2.14
			SS between subsamples			1.798				
			SS within analysis			0.0210				
			F-ratio calculated			95.48				
			F-ratio critical (5% level)			3.02				
					Laboratory 6					
A	1.49	1.71	1.77	1.34	1.86	1.80	1.43	1.51	1.66	1.57
B	1.14[a]	1.74	1.77	1.37	1.83	1.74	1.37	1.68	1.74	1.49
			SS between subsamples			0.4973				
			SS within analysis			0.0258				
			F-ratio calculated			19.27				
			F-ratio critical (5% level)			3.18				

[a]Deleted from statistical calculations as an outlier.

blended and stored in tightly sealed glass containers until analysis. The analyses were made using ASTA method 16.0 and only one determination was made of each subsample. Any deviation from the protocol was to be reported.

Table 4 lists the data reported and the statistical analysis as recommended by Youden (13).

Discussion

The data of Study 1 show that at one extreme the range of the steam-distillable volatile oil in the 10 subsamples of lot analyzed by laboratory 5 is 1.1–3.8 mL/100 g at one standard deviation. At the other extreme, the range is 0.99–1.49 mL/100 g at one standard deviation, reported by laboratory 2. With such a large dispersion of the variation in these examples, one should question whether statistics of normally distributed populations are indicated in this case. The industry refers to taking a "representative" sample without defining what "representative" is. The square root of the number of units in the lot with a maximum of 10 subsamples does not constitute a satisfactory definition.

The data from Study 2 clearly show that no uniform method was used to prepare the subsample for analysis. Those laboratories equipped with mills to reduce the subsample to a U.S. 40 mesh size had little trouble; others experienced great difficulty and resorted to either blending the oversized particles back into the subsample or discarding them.

From the published literature it appears that no in-depth studies have been made on the effect of particle size of the analytical aliquot on the reproducibility of the method. MRI (10) reported running analyses on samples of Korintj and Batavia cassia that had been sieved. Two fractions were studied, that which passes through a U.S. 270 mesh screen and that which would not pass through a U.S. 200 mesh screen. They reported no significant difference in the volatile oil content. It did not appear that their work addressed the question of sample preparation of unprocessed cassia bark.

Study 3 demonstrates that grinding the subsample to pass a U.S. 20 mesh screen is desirable to minimize the loss of volatile oil, which results from excessive heating in the mill, particularly if the condition of the mill is less than optimum.

From a comparison of the S_d values of Studies 2 and 3, it is evident that the repeatability of the steam volatile oil analysis for cassia bark can be improved by careful attention to sample preparation. At least 99% of the aliquot taken from

Table 2. Study 1: One-way analysis of variance of differences between means of treatment groups[a]

Lab.	Mean, mL/100 g	Between-sub. SS[b]	Within-dup. SS[c]	F_c[d]	F(calc.)
1	2.77	0.7645	0.0945	3.18	8.09
2	1.24	0.6260	0.0233	3.02	2.99
3	1.99	0.8760	0.0580	3.02	16.7
4	2.04	0.6050	0.0790	3.02	8.50
5	2.48	1.798	0.0210	3.02	95.5
6	1.62	0.4973	0.0258	3.18	19.3

[a]Groups consist of 10 subsamples. Each subsample within a group is considered a treatment.
[b]Sum of squares of difference between subsamples.
[c]Sum of squares of difference between duplicates.
[d]Critical F-ratio at the 95% confidence level.

the samples for analysis must be ground to pass a U.S. 20 mesh screen. Great care must be exercised to keep the mill in good working condition to minimize undue heating of the sample in the mill.

In addition, it is evident that the repeatability of steam-distillable volatile oil analysis of cassia bark can be improved by closer attention to the principles of good volumetric analysis techniques such as calibrating traps, running xylene blanks, and reading the traps at a standard temperature.

In conclusion, it is necessary that we adjust our expectations to be more commensurate with the limitations of the method as applied to the analysis of cassia bark and accept the fact that we are not justified in reporting analytical values to more than one decimal place. This in itself would be a significant step toward better agreement between laboratories.

Of equal or perhaps more importance is the recognition that lots of cassia bark are not homogeneous with respect to their volatile oil content. The sampling error must be addressed by establishing a sampling plan that will give the analysis a predictable level of confidence. If the analytical report does not reflect the quality of the lot within confidence limits agreed to by the industry, the effort put into this analytical method has little value.

It would appear that our problem is one of establishing these confidence limits. There are 3 possibilities: First, establish sampling constants as given by Gy (14) and Engamells (15). This approach is probably not economically feasible because at least 4 parameters concerning particle geometry, liberation factors, composition factors, and particle size distribution factors for at least 4 varieties of cassia bark would have to be studied in depth. Second, adopt the Monte Carlo system described by Whitaker (16). The difficulty with this system is that it requires large subsamples if the analyte is highly segregated within the lot.

Third, use the sampling system called the 3 Class Attribute Plan (17) designed specifically for the case when one does not have prior knowledge of the functional form of the quality distribution. This is the dilemma that the spice trade faces. Proper application of the principles of 3 class attributes would establish the confidence limits alluded to above.

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Table 3. Study 2: Data and statistical analysis for screening subsample of cassia bark

Lab.	Spl. 1 mL/100 g	Spl. 2 mL/100 g	Mill[a]	Screening procedure
1	2.60	2.57	CF	Sample 1:60% thru U.S. 30 mesh Sample 2:100% thru U.S. 30 mesh and 75% thru U.S. 40 mesh, entire sample blended
2	2.46	2.56	CF	100% thru U.S. 40 mesh
3	3.06	2.98	CW	100% thru U.S. 40 mesh
4	2.77	2.35	CW	100% thru 0.5 mm (0.02 in.) screen in mill
5	2.57	2.62	H	100% thru 0.020 in. (0.5 mm) screen in mill
6	2.90	3.00	H	100% thru U.S. 40 mesh
7	3.04	3.07	H	200 g aliquot from sample 1 and 2; 97% thru U.S. 60 mesh; 3% overs blended in with 60 mesh
8	1.57	2.00	CW	100% thru 10 mesh 100 g aliquot thru U.S. 40
9	3.03	3.06	CW	100% thru 2 mm screen in mill Sample 1:78% thru 9.5 mm screen in mill, overs discarded Sample 2:76% thru 0.5 mm screen in mill, overs discarded
Mean[b]	2.667	2.690		

[a]H = hammer mill (Weber & White type);
CW = cutting mill (Wiley-Thomas type);
CF = cutting mill (Fritzpatrick type).
[b]S_r = random error = 0.1590;
S_d = combined effect of systematic error and random error = 0.575;
$F = S_d^2/S_r^2 = 6.25$;
F_c = 3.79 at 95% confidence level.

Table 4. Study 3: Data and statistical analysis on effect of screen size

Lab.	Volatile oil, mL/100 g	
	20 Mesh screen	40 Mesh screen
1	2.57	2.34
2	2.70	2.74
3	2.69	2.49
4[a]	3.23	3.23
5	2.60	2.63
6[b]	2.74	3.63
7	2.26	2.34
8[c]	2.68	3.34
9	2.34	2.28
Mean[d]	2.527	2.470

These results were deleted from the statistical analysis for the following reasons:

[a]Laboratory 4 results had an excessive difference between the total of the pairs and the average total difference of all results.

[b]Laboratory 6 results had an excessive difference between pairs that exceeded 2.5 times the average absolute difference of all values.

[c]Laboratory 8 reported they did not use the same mill to prepare each analytical sample.

[d]S_r = random error = 0.093;
S_d = combined effect of systematic error and random error = 0.24;
$F = S_d^2/S_r^2 = 6.7$;
F_c = 5.05 at the 95% confidence level.

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MICROBIOLOGICAL METHODS

Gas Chromatographic Detection of D-(−)-2,3-Butanediol and Butyric Acid Produced by Sporeformers in Cream-Style Corn and Canned Beef Noodle Soup: Collaborative Study

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A gas chromatographic method that identifies sporeformers as the cause of spoilage in swollen cans of low-acid foods was collaboratively studied in 2 stages. Two organic compounds produced by sporeformers, D-(−)-2,3-butanediol and butyric acid, are measured in the upper phase after centrifugation of the liquid portion of the can contents. Each sample is assayed on 2 packed columns designed for the assay of aqueous solutions of volatile fatty acids, using flame ionization detectors. For study 1, 16 duplicate inoculated cans of cream-style corn and beef noodle soup were sent to 9 collaborators. For study 2, 7 collaborators received 11 duplicate inoculated cans of the 2 foods. Duplicate uninoculated cans of each food served as negative controls. The inocula were 6 sporeforming organisms (4 *Clostridium* and 2 gas-forming *Bacillus* species) and 2 nonsporeformers. After the deletion of marginal samples, the percentages of correctly identified sporeformers and nonsporeformers in beef noodle soup were 83 (110/132) and 90 (54/60), respectively; corresponding percentages for cream-style corn were 80 (98/123) and 100 (35/35). The method has been adopted official first action.

This report describes the collaborative study of a gas chromatographic (GC) method using flame ionization detection (FID) for identifying 2 compounds produced by sporeforming organisms in swollen cans of low-acid foods. The absence of these 2 compounds in microbial spoilage with the presence of gas production indicates spoilage by nonsporeformers. The method was developed and evaluated (1) for use as a rapid screening technique to assess the potential health hazard of swollen cans of low-acid foods that contain gas as a result of microbial activity but no longer contain viable organisms. It is not uncommon to find "autosterilized" cans. No method is currently available to evaluate the cause of microbial spoilage in such sterile swollen cans. In practice, swollen cans are routinely examined for the presence of microorganisms. Then the potential health hazard is estimated by ascertaining whether the microbial contamination resulted from underprocessing or from postprocessing contamination caused by seam leakage. Although there are exceptions, it is common practice to assume that the presence of sporeformers alone is indicative of underprocessing, whereas a mixture of sporeformers and nonsporeformers, or the presence of nonsporeformers alone, is indicative of can leakage.

The method used in this study detects 2 metabolic products formed by sporeforming organisms, but not formed by nonsporeformers, growing in low-acid foods. The presence of either of 2 organic compounds, D-(−)-2,3-butanediol (referred to as X_6 during method development but hereafter as BD) or butyric acid (referred to as X_{11} during method development but hereafter as BA), in the can contents of a swollen can of low-acid food indicates that growth of a sporeforming organism has occurred. The absence of both compounds in microbial spoilage with gas production indicates that growth of a nonsporeformer has occurred. The production of gas in an inoculated can is assumed to be the result of microbial activity.

Collaborative Study

The method for the identification and assay of the 2 organic compounds produced by sporeformers growing in low-acid foods was collaboratively studied with inoculated cans of 2 foods. For study 1, 9 analysts assayed 16 inoculated cans in duplicate; for study 2, 7 analysts assayed 11 inoculated cans in duplicate.

Cans of cream-style corn (corn) and beef noodle soup (BNS) with identical can codes were purchased on the local market and inoculated with one of the following: 2 strains of *Clostridium sporogenes* (3679 and PA 16), *Clostridium butyricum, Clostridium thermosaccharolyticum, Bacillus polymyxa, Bacillus macerans, Escherichia coli, or Pseudomonas aeruginosa*. Each inoculated can was sealed in a polyethylene bag, held at temperatures ranging from 35 to 55°C until gas production occurred, and stored at 5°C for 1–2 months before shipment to the collaborators. Each inoculated can swell was contained in a vacuum-sealed double bag when shipped at 5–15°C. The analysts were instructed to report the cause of spoilage in each sample as that from either a sporeformer or a nonsporeformer. The correct identification for each sample was based on comparison with the organism used to inoculate the can. For the preparation of standards and for an assay prior to each sample assay, it was suggested that the analysts use water that elutes no detectable compounds on the 2 stationary phases used for the assay of samples.

Sporeformers in Low-Acid Canned Foods

Gas Chromatographic Method

First Action

46.A01 ***Principle***

Two org. compds produced by sporeforming organisms in low-acid canned foods, D-(−)-2,3-butanediol (BD) and butyric acid (BA), but not produced by nonsporeformers, are measured by gas chromatgy. Identification of BD and BA is based on relative retention times (RRTs) to internal std, propionic acid. Identification of sporeforming organisms as cause of spoilage is based on ratio of peak hts for BD and butyric acid (in external std) and BA and butyric acid (in external std).

46.A02 ***Apparatus***

(a) *Gas chromatograph.*—Suitable for use with 2 heated flash vaporizer injectors contg glass sleeves; equipped with flame ioni-

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The recommendation of the Associate Referee was approved by the General Referee and Committee F and was adopted by the Association. See the General Referee and Committee reports, *J. Assoc. Off. Anal. Chem.* (1985) **68**, March issue.
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zation detectors (FID); linked to data processor (if available) with printer/plotter (Sigma Series Instrument with console and printer/plotter, Perkin-Elmer, or equiv.).

(b) *Gas chromatographic columns.*—(*1*) 1.8 m (6 ft) × 2 mm id glass column packed with 15% SP 1220/1% H_3PO_4 on 100–120 mesh Chromosorb W(AW); (*2*) 1.8 m × 2 mm (id) glass column packed with 0.3% CW 20M/0.1% H_3PO_4 on 60–80 mesh Carbopack C (Supelco Inc.). Condition both columns, using recommended procedures: purge column at ambient temp. ≥30 min with carrier gas at 20 mL/min; then program from 50° to 150° at 2°/min and hold overnight. Cool column 1, attach to detector, and set column temp. at 118°. Inject twenty 10 μL portions of freshly boiled H_2O on column 2 at 150°. Then cool, attach to detector, and set column temp. at 125°.

(c) *Operating conditions.*—Select 2 methods from available chromatgc data systems software (if data processor is available) that provide relative retention time (RRT) = 1.00 for internal std. Column 1: injector 200°, detector 240°, column 118° for 12 min isothermal run; He carrier flow rate 24 mL/min; propionic acid elution time 2.70–3.30 min; electrometer range 10, attenuation 4; chart speed 5 mm/min; injection vol. 1 μL. For butyric acid, retention time = ca 6 min, RRT = 1.7, and peak ht = 60% FSD (10 cm). Column 2: injector 200°, detector 240°, column 125° for 25 min isothermal run; N carrier flow rate 15 mL/min; propionic acid elution time 2.25–2.45 min; electrometer range 10, attenuation 2; chart speed 10 mm/min; injection vol. 0.3 μL. For butyric acid, retention time = ca 6.5 min, RRT = 2.7, and peak ht = 60% FSD (10 cm). Theoretical plates for each column ≥1600.

(d) *Syringes.*—1 and 5 μL (Hamilton 7001-N and 1705-N, or equiv.).

(e) *Centrifuge.*—With adapters suitable to accept 5 mL minivials, 1.0 and 5.0 mL capacity, with silicone stopper and screw cap

Table 1. Collaborative results of GC detection of cause of spoilage in inoculated cans (duplicates) of low-acid foods: Study 1

Inoculum	Laboratory									No. correct/total	Percent correct
	1	2	3	4	5	6	7	8	9		
Cream-Style Corn											
Sporeformers:											
C. sporogenes 3679	S	S	S	S	S	S	S	—[a]	S	15/15	100
	S	S	S	S	S	S	S	—[a]	—[b]		
C. sporogenes PA 16	S	NS	S	S	S	S	S	S	S	16/18	89
	S	S	S	S	S	S	S	S	NS		
C. thermosaccharolyticum[c]	NS	NS	NS	NS	NS	NS	—[b]	NS	—[b]	0/14	0
	NS	NS	NS	NS	NS	NS	—[b]	NS	—[b]		
C. butyricum	S	NS	NS	S	S	S	S	NS	NS	8/18	44
	S	NS	NS	NS	NS	S	S	NS	NS		
B. polymyxa 7047	S	NS	S	S	S	S	S	S	S	15/18	83
	S	NS	S	S	S	S	NS	S	S		
B. macerans 843	S	NS	S	S	S	S	S	S	NS	13/18	72
	S	NS	S	S	S	NS	S	S	NS		
No. correct/total	10/12	3/12	8/12	9/12	9/12	9/12	9/10	6/10	4/9	67/101	66
Percent correct	83	25	67	75	75	75	90	60	44		
Nonsporeformers:											
Ps. aeruginosa	NS	NS	NS	NS	NS	NS	NS	NS	NS	18/18	100
	NS	NS	NS	NS	NS	NS	NS	NS	NS		
E. coli	NS	NS	NS	NS	NS	NS	NS	NS	NS	17/17	100
	NS	NS	NS	NS	NS	NS	NS	NS	—[b]		
No. correct/total	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	3/3	35/35	100
Percent correct	100	100	100	100	100	100	100	100	100		
Beef Noodle Soup											
Sporeformers:											
C. sporogenes 3679	S	S	S	S	S	S	S	S	S	18/18	100
	S	S	S	S	S	S	S	S	S		
C. sporogenes PA 16	S	S	S	S	S	S	S	S	S	18/18	100
	S	S	S	S	S	S	S	S	S		
C. thermosaccharolyticum[c]	NS	NS	NS	NS	NS	NS	S	NS	NS	1/18	6
	NS	NS	NS	NS	NS	NS	NS	NS	NS		
C. butyricum	S	S	S	S	S	S	S	S	S	18/18	100
	S	S	S	S	S	S	S	S	S		
B. polymyxa 7047	S	NS	NS	NS	NS	NS	NS	NS	NS	1/18	6
	NS	NS	NS	NS	NS	NS	NS	NS	NS		
B. macerans 843[c]	NS	NS	NS	NS	S	NS	S	S	NS	4/18	22
	NS	NS	NS	NS	S	NS	NS	NS	NS		
No. correct/total	7/12	6/12	6/12	6/12	8/12	6/12	3/12	7/12	6/12	60/108	56
Percent correct	58	50	50	50	67	50	67	58	50		
Nonsporeformers:											
Ps. aeruginosa	NS	S	S	NS	S	NS	S	NS	NS	13/18	72
	NS	NS	NS	NS	NS	NS	S	NS	NS		
E. coli	NS	NS	NS	NS	NS	NS	NS	NS	NS	18/18	100
	NS	NS	NS	NS	NS	NS	NS	NS	NS		
No. correct/total	4/4	3/4	3/4	4/4	3/4	4/4	2/4	4/4	4/4	31/36	86
Percent correct	100	75	75	100	75	100	50	100	100		

NS = spoilage caused by a nonsporeforming organism.
S = spoilage caused by a sporeforming organism.
[a]No sample sent to collaborator.
[b]Collaborator had problems with sample cleanup.
[c]Marginal samples deleted in Table 4.

Table 2. Collaborative results of GC detection of cause of spoilage in inoculated cans (duplicates) of low-acid foods: Study 2

Inoculum	Laboratory 1	2	4	5	6	8	No. correct/ total	Percent correct
Cream-Style Corn								
Sporeformers:								
C. thermosaccharo-	S	S	NS	S	S	NS	9/12	75
lyticum	S	S	NS	S	S	S		
C. butyricum	S	NS	S	S	S	S	11/12	92
	S	S	S	S	S	S		
B. macerans	S	S	S	S	S	NS	11/12	92
	S	S	S	S	S	S		
No. correct/total	6/6	5/6	4/6	6/6	6/6	4/6	31/36	86
Percent correct	100	83	67	100	100	67		
Beef Noodle Soup								
Sporeformers:								
C. sporogenes 3679	S	S	S	S	S	S	12/12	100
	S	S	S	S	S	S		
C. sporogenes PA 16	S	S	S	S	S	S	12/12	100
	S	S	S	S	S	S		
C. thermosaccharo-	S	S	S	S	S	S	12/12	100
lyticum	S	S	S	S	S	S		
C. butyricum	S	S	S	S	S	S	12/12	100
	S	S	S	S	S	S		
B. polymyxa[a]	NS	S	NS	NS	S	NS	4/12	33
	NS	S	NS	NS	S	NS		
B. macerans	S	S	S	NS	S	NS	7/12	58
	NS	S	S	S	NS	NS		
No. correct/total	9/12	12/12	10/12	9/12	11/12	8/12	59/72	82
Percent correct	75	100	83	75	92	67		
Nonsporeformers:								
Ps. aeruginosa	NS	NS	NS	NS	NS	NS	12/12	100
	NS	NS	NS	NS	NS	NS		
E. coli	NS	S	NS	NS	NS	NS	11/12	92
	NS	NS	NS	NS	NS	NS		
No. correct/total	4/4	3/4	4/4	4/4	4/4	4/4	23/24	96
Percent correct	100	75	100	100	100	100		

S = spoilage by a sporeforming organism.
NS = spoilage by a nonsporeforming organism.
[a]Marginal samples deleted in Table 4.

(95010 and 95050, Alltech Associates, Inc., 2051 Waukegan Rd, Deerfield, IL 66015), or equiv.

(f) *Disposable Pasteur pipets.*—7760-F 30 series (Arthur H. Thomas Co.), or equiv.

46.A03 *Reagents*

(a) *Water.*—Distd H_2O that elutes with no detectable peaks on 2 columns used for assay.

(b) *External std solns.*—(*1*) For column 1, WSFA-2 (Supelco Inc.), or equiv. It must contain aq. soln of propionic, isobutyric, and butyric acids. Concn of butyric acid should be 0.1%. Concns of propionic and isobutyric acids should be sufficient to produce peak hts ranging from detectable to 100% scale deflection (SD).

(*2*) For column 2, dil. WSFA-2 with equal vol. H_2O or prep. equiv. soln. It must contain aq. soln of propionic and butyric acids. Concn of butyric acid should be 0.05%. Concn of propionic acid should be sufficient to produce peak ht ranging from detectable to 100% SD.

(c) *Internal std solns.*—(*1*) For column 1, dil. 5 μL reagent grade propionic acid to 5 mL with H_2O. For injection, take up 1 μL H_2O into 5 μL syringe, followed by 0.1 μL soln. Modify proportions of 2 components, if necessary, to give ca 50% FSD for propionic acid peak eluting in 2–3 min. (*2*) For column 2, dil. portion of internal std for column 1 with equal vol. H_2O. For injection, take up 0.3 μL H_2O into 1 μL syringe, followed by 0.02 μL of this diln. Modify proportions of 2 components, if necessary, to give ca 50% FSD for propionic acid peak eluting in 2–3 min.

46.A04 *Assay*

Pierce each can aseptically by any microbiologically acceptable technic (see **46.065**(a), (c) and **46.066**). Transfer portion of liq. can contents to 5 mL mini-vial, using sterile Pasteur disposable pipet, or equiv., and store at ca −20° overnight or longer. Warm 5 mL vials to room temp. and then centrf. 15 min at 1000 ×*g* or until phases sep. Transfer portion of clear upper phase to 1 mL mini-vial, using sterile Pasteur disposable pipet, or equiv. Store this upper phase at −20° overnight or until day of assay.

Let 1 mL mini-vials of upper phase of centrfd can contents warm to room temp. Use syringe to remove (and discard) any particulate matter from bottom of cone; then mix sample, using syringe. For assays on either column, assay external std to ensure optimal instrumental conditions; propionic acid must be eluted in time range such that RRT = 1.000; theoretical plates should be ca ≥1600; and butyric acid peak should be ca 60% FSD. Then replace glass liner in injector and inject sample of H_2O, using sample syringe. Repeat H_2O injections, if necessary, until chromatogram has no ghost peaks. For assays on column 1, take up 1 μL portion of sample into 5 μL syringe, followed by 0.1 μL internal std soln 1 and inject into gas chromatograph. For assays on column 2, take up 0.3 μL portion of sample into 1 μL syringe followed by 0.02 μL internal std soln 2 and inject into gas chromatograph.

46.A05 *Interpretation*

For each sample, examine chromatograms from both columns, together with chromatograms for external std assayed same day. On column 1, BD elutes as tailing peak with retention time between those for isobutyric and butyric acids; it will not elute on column 2 in 25 min run. Sample contg BA gives peak eluting in both sample chromatograms in retention time range for butyric acid. Measure peak ht for BD peak and divide by peak ht for butyric acid in external std 1 assayed on column 1. Measure BA peak ht from column 2 and divide by peak ht for butyric acid in external std 2

assayed on column 2. Peak ht ratio for BD ≥0.39 or peak ht ratio for BA ≥0.30 indicates sporeformers as cause of spoilage. Record cause of spoilage as from either sporeformer or nonsporeformer.

Results and Discussion

The critical peak height ratios for BD and BA used to identify sporeformers as the cause of spoilage are based on all available assay results (both published and unpublished) collected during the development and evaluation of the method (1). These results represent assays of a total of 55 cans of corn and 27 cans of BNS inoculated with the same microorganisms as those used for the collaborative study. Data from these assays were used for a discriminant analysis (2). A line joining the points (BD = 0.39, BA = 0) and (BD = 0, BA = 0.30) was found to yield the best discrimination between sporeformers and nonsporeformers.

Table 1 lists the results for study 1. Each analyst was sent duplicate cans of each food inoculated with 1 of each of the 8 strains of microorganisms along with duplicate negative controls (uninoculated cans) of each food. Inadvertently, analyst 8 received 4 cans of corn inoculated with *C. thermosaccharolyticum* and no cans inoculated with *C. sporogenes* 3679. The analyst reported nonsporeformers as the cause of spoilage for all 4 cans. For statistical analysis, 2 of these 4 results were deleted. The overall percentage of correct identifications for the inoculated cans in study 1 was 69 (193/280). The percentage of correct identifications for the corn samples was 75 (102/136) and that for the BNS was 63 (91/144). None of the 14 cans of corn for which results were reported and only 1 of the 18 cans of BNS inoculated with *C. thermosaccharolyticum* were identified correctly. Of the cans inoculated with *C. butyricum*, only 8 of the 18 cans of corn but all of the BNS cans were correctly identified. Of the cans inoculated with *B. polymyxa*, 15 of the 18 cans of corn (83%) and only 1 of the 18 cans of BNS were identified correctly. All of the negative controls were reported as negative (i.e., no peaks above background noise were observed in the RRT (relative retention time) ranges for BD and BA). The overall percentage of correct identifications reported for the 5 low-acid canned foods used in the method development (1) was 84.

For study 2, each analyst received duplicate cans of BNS inoculated with each of the 8 strains of microorganisms and duplicate cans of corn inoculated with one of the following: *C. butyricum*, *C. thermosaccharolyticum*, or *B. macerans*. The results of this study are shown in Table 2. One collaborator inadvertently deviated from the procedure, and these data were not included in the statistical analysis. The overall percentage of correct identifications for study 2 was 86 (113/132) with a range of 80–92% for the 95% confidence interval (3). The percentages of correct identifications for BNS were 33 for *B. polymyxa* and 58 for *B. macerans*. The overall percentages of correct identifications were 85 (82/96) for the inoculated cans of BNS and 86 (31/36) for the inoculated cans of corn. None of the chromatograms for the uninoculated cans contained peaks above background noise in the RRT ranges for BD and BA.

For both studies, samples of each food were inoculated and held 1–2 months before the anticipated shipping date. Some of the cultures, i.e., *C. thermosaccharolyticum* and both *Bacillus* species used for study 1, did not grow as they did for the method development and evaluation. Tests on replicates of these sets of samples used in studies 1 and 2 indicated some production of BA and BD, but in concentrations that were insufficient for the cause of spoilage to be identified by the established criteria as a sporeforming organism. We assumed that the additional holding time in the collaborators' laboratories would allow additional growth but, as the data in Table 3 indicate, that assumption was incorrect. Table 3 compares the analyses performed before or after the samples were shipped with the analyses by the collaborators. For 4 sets of replicates the assays were known to be negative

Table 3. Comparison of analyses before or after samples were shipped with analyses by collaborators

	Study 1								Study 2							
	Beef noodle soup				Cream-style corn				Beef noodle soup				Cream-style corn			
	Anal. before or after shipment				Anal. before or after shipment				Anal. before or after shipment				Anal. before or after shipment			
Inoculum	BD pk ht ratio	BA pk ht ratio	ID[a]	% correctly identified by colls[b]	BD pk ht ratio	BA pk ht ratio	ID	% correctly identified by colls	BD pk ht ratio	BA pk ht ratio	ID	% correctly identified by colls	BD pk ht ratio	BA ht pk ratio	ID	% correctly identified by colls
C. sporogenes 3679	—[c]	>1.00	S[d]	100	—	>1.00	S	100	—	>1.00	S	100				
	—	>1.00	S[e]		—	0.78	S		—	0.75	S					
	—	>1.00	S		—	0.92	S		—	>1.00	S					
C. sporogenes PA 16	—	>1.00	S	100	—	0.96	S	89	—	0.51	S	100				
	—	0.10	NS[f]		—	—	NS[f]		—	>1.00	S		—	0.45	S	
C. thermosaccharolyticum	—	0.16	NS[f]	6	—	—	NS[f]	0	—	>1.00	S	100	—	0.47	S	75
	—	>1.00	S		—	>1.00	S		—	>1.00	S		—	0.53	S	
C. butyricum	—	0.63	S	100	—	>1.00	S	44	—	>1.00	S	100	—	0.71	S	92
	0.14	1.00	S		>1.00	—	S		0.29	—	NS[f]					
B. polymyxa	0.28	—	NS	6	>1.00	—	S	83	0.22	—	NS[f]	33				
	0.10	—	NS[f]		>1.00	—	S		0.48	—	S		>1.00	—	S	
B. macerans	0.13	—	NS[f]	22	>1.00	—	S	72	0.23	—	NS	58	>1.00	—	S	92
	—	—	NS		—	—	NS		—	—	NS					
Ps. aeruginosa	—	—	NS	72	—	—	NS	100	—	—	NS	100				
	—	—	NS		—	—	NS		—	—	NS					
E. coli	—	—	NS	100	—	—	NS	100	—	—	NS	92				

[a]ID = cause of spoilage, based on analysis before or after shipment. S = spoilage by a sporeforming organism. NS = spoilage by a nonsporeforming organism.
[b]Based on comparison with organism used to inoculate can (as shown in Tables 1 and 2).
[c]— = none detected by GC analysis.
[d]Analysis performed before shipment of samples.
[e]Analysis performed after shipment of samples.
[f]Sample known (before shipment) to produce peak height ratios of <0.30 (BA) and <0.39 (BD).

or marginal prior to shipment. The collaborators incorrectly identified 85% of the samples in these 4 sets.

The collaborators reported the cause of spoilage (gas production) in each can swell as that from either a sporeforming organism or a nonsporeformer. These binomial results cannot be used to compute interlaboratory and intralaboratory estimates of variation. Tables 1–3 summarize the data in terms of correct identifications based on comparisons with the organisms used to inoculate the can. Tables 1 and 2 also show the percentages of sporeformers and nonsporeformers that were correctly reported by each analyst for each set of samples.

Table 4 presents a summary of the percentages of correctly identified samples, using all sets of samples sent to collaborators and deleting the sample sets for which preshipment assays gave concentrations of BD and BA that were less than the levels required for identification of sporeformers. The percentages of correctly identified sporeformers and nonsporeformers were computed for all the sets of samples sent to the collaborators for corn and BNS. These data indicate that samples inoculated with nonsporeformers had significantly higher percentages of correct identifications (94 vs 66%) than samples inoculated with sporeformers. The hypothesis that the percentages are equal was tested by assuming that the normal distribution is a valid approximation (3) to the binomial distribution. When the 4 marginal sets of data (replicate cans which, after inoculation with sporeformers, were found to be negative for spores on the basis of GC analysis or produced peak height ratios of <0.39 for BD and <0.30 for BA before shipment to collaborators) are deleted, the percentage of correctly identified sporeformers does not differ (at $\alpha = 0.05$ level) from that for nonsporeformers (83% (110/132) vs 90% (54/60), respectively) for the combined BNS data from studies 1 and 2. Similarly, the overall percentages of correctly identified sporeformers and nonsporeformers in analyses by collaborators (81 vs 94%, respectively) are improved, although they are still significantly different (at $\alpha = 0.05$ level), after deletion of the marginal data. The percentages of correctly identified sporeformers and nonsporeformers in corn are 80 (98/123) and 100 (35/35), respectively, with the marginal data deleted.

Chromatograms for the external standard demonstrated instrumental problems (possible plumbing leaks) at the time Collaborator 2 assayed the study 1 samples; Table 1 shows that Collaborator 2 misidentified all 4 samples of corn inoculated with the 2 *Bacillus* species. The problem had been resolved when the study 2 samples were assayed; Collaborator 2 correctly identified all 6 cans of the 2 foods inoculated with the 2 *Bacillus* species for study 2 (Table 2).

Most of the collaborators reported difficulty with the preparation of a clear upper phase after centrifugation of the can contents for the cream-style corn used in study 1. The problem occurred with both uninoculated and inoculated cans. Increasing the centrifugation time usually provided a sample suitable for assay. One collaborator was unable to assay 2 of the 16 corn samples because of emulsion problems; another omitted 4 of the 16 assays. All collaborators reported data for all samples in study 2. Those collaborators with little experience with GC reported problems in handling the 1 μL syringe. Analysts trying to use on-column injection systems had problems with ghost peaks.

With natural swells that are sterile, an analyst using this method can consider a sample as suspect for spoilage by sporeforming organisms if either BD or BA is present above the detection limit, which is defined as that quantity which produces a signal that is twice the size of the standard deviation of the background. To assess the significance of peak heights that are greater than those produced by quantities above the detection limit but less than the peak heights required for identification of spoilage by a sporeformer, the analyst must observe all of the built-in checks for the identification of these compounds and eliminate the possibility of syringe contamination. An external standard must be assayed each day that samples are assayed; an injection of water must precede each sample injection; the BD must appear as a tailing peak in the column 1 chromatogram with a well defined RRT and must not elute from column 2 during the 25 min assay; the BA must produce a symmetrical peak and have an RRT in the range for butyric acid on both columns.

Measurable amounts of BD and BA were not observed by the collaborators in the 60 negative controls (the uninoculated cans of the 2 foods) nor in the uninoculated cans assayed during the development and evaluation of the method (1).

No data are available that correlate the growth of sporeforming organisms with the production of BD and BA. Using the line joining the points (BD = 0.39, BA = 0) and (BD = 0, BA = 0.30), which is based on data from inoculated cans, the collaborators reported nonsporeformers as the cause of spoilage for samples that produced BD and BA peaks with heights ranging from 1 to 3.4 cm. For the study 1 data, 27/34 misidentified sporeformers in samples of corn and 25/48 misidentified sporeformers in samples of BNS produced peak heights for BD and BA that were within this range.

The concentration of BA or BD in an inoculated can that is required to identify a sporeformer, i.e., the amount that gives a peak height ratio ≥0.30 or ≥0.39, respectively, can be estimated from the peak height for butyric acid in the appropriate external standard. External standard 2 contains 0.05% butyric acid. Thus the upper phase that is present after centrifugation of the liquid contents of the can must contain ≥0.015% BA (0.015/0.05 = 0.30). A 0.1% (v/v) solution of D-(−)-2,3-butanediol in water produces a tailing peak in the

Table 4. Percentages of correct identifications calculated with and without deletion of marginal samples

		With all data		With samples deleted[a]	
Study	Group	Sporeformers	Nonsporeformers	Sporeformers	Nonsporeformers
1	Cream-style corn	66 (67/101)[b]	100[c] (35/35)	77(67/87)	100[c](35/35)
2	Cream-style corn[d]	86 (31/36)	—	86(31/36)	—
1	Beef noodle soup	56 (60/108)	86[c] (31/36)	76(55/72)	86 (31/36)
2	Beef noodle soup	82 (59/72)	96[c] (23/24)	92(55/60)	96 (23/24)
Overall average		66 (186/281)	94[c] (89/95)	81 (177/219)	94[c] (89/95)

[a]All samples deleted were found by GC analysis to be negative for spores or produced peak height ratios of <0.39 for BD and <0.30 for BA before shipment to collaborators.
[b]Number of correct identifications/number of observations in parentheses.
[c]Difference between sporeformers and nonsporeformers significant at $\alpha = 0.05$ level.
[d]Samples of nonsporeformers were not sent to collaborators. This group of samples was not used in the calculation of the overall averages.

column 1 chromatogram with a peak height that is about the same as that for external standard 1, which contains 0.1% butyric acid. Thus the upper phase that is present after centrifugation of the liquid can contents must contain ≥0.039% BD (0.039/0.1 = 0.39).

The results from study 1 indicate that the overall percentage of correct identifications is 69, which is less than the 84% obtained in the method development (1). This lower overall percentage is due in part to cultures that did not grow in the inoculated cans (Table 3). If the marginal samples are excluded (Table 4), the overall percentage of correct identifications for study 1 is 82.

Nonetheless, a second study was conducted; the overall percentage of correct identifications for study 2 is 91 with the marginal samples excluded.

Since the absence of a critical peak in the chromatogram is an indication that a sporeformer is not the cause of spoilage, it is not surprising that for both studies 89 of 95 (94%) nonsporeformers were correctly identified compared with 217 of 317 (68%) sporeforming organisms (marginal samples included). The comparison is more favorable (96 vs 83%, respectively) if only data from study 2 are considered (marginal samples included).

Recommendation

The overall percentages of correct identifications obtained for study 1 (82%) and study 2 (91%) do not differ significantly from the 84% obtained for the published method (1). Therefore, it is recommended that the method be adopted official first action.

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MYCOTOXINS

Quantitative Liquid Chromatographic Method Using Fluorescence Detection for Determining Zearalenone and Its Metabolites in Blood Plasma and Urine

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The liquid chromatographic (LC) method described, suitable for use with both blood plasma and urine, is applicable for determination of zearalenone and α-zearalenol at levels as low as 0.5 ng/mL plasma and 5 ng/mL urine. The sample is incubated overnight with β-glucuronidase to analyze for both conjugated and unconjugated forms of zearalenone. The next day, the sample is acidified with H_3PO_4, extracted with chloroform, and evaporated to dryness. The residue is dissolved in toluene and loaded onto a silica gel cartridge which is washed with toluene and eluted with toluene–acetone (88 + 12). The eluate is evaporated, and the residue is dissolved in chloroform, extracted with 0.18M NaOH, neutralized with H_3PO_4, and re-extracted with chloroform. The chloroform extract is evaporated, dissolved in mobile phase for LC, and injected onto a normal phase column under the following chromatographic conditions: mobile phase of water-saturated dichloromethane containing 2% 1-propanol, and fluorescence detector, excitation wavelength 236 nm, and 418 nm cut-off emission filter. Recoveries of zearalenone and its metabolites from blood plasma and urine are 80–89% in the range 2.0–10 ng standard/mL plasma, and 81–90% in the range 10–30 ng standard/mL urine. This method was used to analyze blood and urine samples from a pig fed zearalenone-contaminated feed (5 mg/kg), corresponding to 80 μg/kg body weight. Zearalenone was rapidly metabolized to α-zearalenol, which appeared in the blood only 30 min after feeding. Almost all zearalenone and α-zearalenol was found conjugated with glucuronic acid in both blood plasma and urine.

Several species of *Fusarium* fungi are capable of producing zearalenone, 6-(10-hydroxy-6-oxo-*trans*-1-undecenyl)-β-resorcylic acid lactone, an estrogenic mycotoxin. Zearalenone is mainly a contaminant in corn, but it may also occur in wheat, barley, oats, sorghum, sesame, and hay (1, 2).

The pig is perhaps the animal most sensitive to zearalenone. A dose of 1 mg zearalenone per day given to a 50 kg gilt is sufficient to cause signs of vulvovaginitis on the 8th day (3).

To conduct pharmacokinetic studies on zearalenone metabolites when zearalenone is given in low doses (1–5 mg/kg in feed), a very sensitive analytical method is needed. This method should also include determination of both free and conjugated forms of zearalenone and its metabolites, because zearalenone is metabolized via 2 pathways—conjugation and reduction to zearalenol (4). Mirocha et al. (5) have investigated the in vivo metabolism of zearalenone in laboratory and farm animals. They found in the urine of a 3-week-old piglet given 1.0 g zearalenone both free and glucuronide-conjugated zearalenone (63%), α-zearalenol (32%), and β-zearalenol (5%).

A sensitive method which can be used to detect both free and conjugated substances is also suitable for detection of zearalenone and its metabolites in blood plasma or urine of animals suspected of ingesting contaminated feed. Recently, 2 sensitive methods for free zearalenone and metabolites in blood have been published, a liquid chromatographic (LC) method by Trenholm et al. (6), and a radioimmunoassay by Thouvenot and Morfin (7). Detection limit for the LC method was 0.6 ng/mL plasma, and for radioimmunoassay was 5 ng/mL serum.

James et al. (8) described a procedure which detects 270 ng zearalenone and 500 ng zearalenol/mL urine, both free and conjugated forms.

In the present paper we describe a sensitive LC method, suitable for use with both blood plasma and urine and which detects both free and conjugated forms of zearalenone. Results are presented from a zearalenone feeding experiment.

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[1]The work reported here is contained in a Ph.D. thesis to be submitted to Department of Zoophysiology, University of Uppsala.

METHOD

Apparatus

(a) *Vortex-mixer.*—Super-Mixer (Lab-Line Instruments, Inc., Melrose Park, IL).

(b) *Centrifuge.*—Wifug × 1 (Wifug, Stockholm, Sweden).

(c) *Liquid chromatography.*—Waters 6000 A solvent delivery system (Waters Associates, Inc., Milford, MA). LC operating conditions: LC column, μPorasil normal phase, 3.9 mm id × 30 cm (No. 27477, Waters Associates); constant solvent flow, 1.2 mL/min; recorder, chart speed 0.5 cm/min (W + W Recorder 1100, W + W Electronic AG, Basel-Münchenstein, Switzerland); fluorescence detector, Kratos Model FS970 Spectrofluoro Monitor with excitation wavelength 236 nm, 418 nm cut-off emission filter, range 0.02 μA (zero set at 0.05 μA), and time constant 3 s (Kratos Analytical Instruments, Ramsey, NJ).

(d) *Rotary evaporator.*—Rotavapor R-110 (Büchi Laboratoriums Technik AG, Flawil, Switzerland).

(e) *Sample concentrator.*—SC 3 (Techne Ltd, Cambridge, UK).

(f) *Cleanup column.*—Sep-Pak silica cartridge (Waters Associates).

(g) *Extraction column.*—Extrelut® (No. 11737, E. Merck).

Reagents

(a) *Solvents.*—Chloroform, toluene, 1-propanol, dichloromethane, acetone, all p.a. grade (E. Merck, Darmstadt, FRG).

(b) *β-Glucuronidase.*—9 mg/50 μL 0.2M sodium acetate buffer (pH 5.5). Enzyme activity 550 000 units/g solid (from bovine liver type B-1, Sigma Chemical Co., St. Louis, MO).

(c) *Sulphatase.*—Enzyme activity 5110 units/mL. Preparation also contains β-glucuronidase 100 000 units/mL (from *Helix pomatia* type H-2, Sigma Chemical Co.).

(d) *LC mobile solvent.*—Water-saturated dichloromethane containing 2% 1-propanol.

(e) *Standards.*—Highly purified zearalenone (99.8%, donated by Commercial Solvents Corp., Terre Haute, IN). α- and β-Zearalenol were made by reduction of zearalenone with $NaBH_4$ (9).

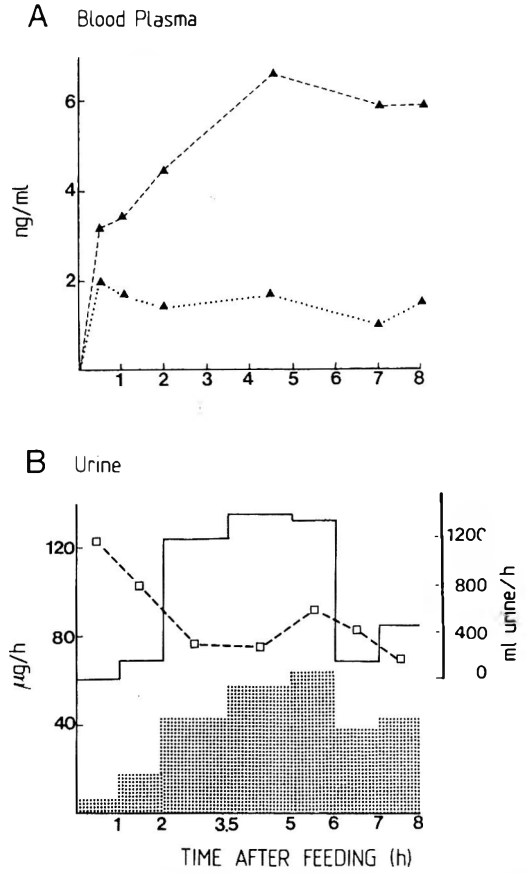

Figure 1. Zearalenone and α-zearalenol content in blood plasma and urine from a swine fed zearalenone-contaminated feed (5 mg/kg diet). (A) Zearalenone (. . . . .) and α-zearalenol (- - - - -) in blood plasma, expressed as ng/mL plasma. (B) Total amount zearalenone + α-zearalenol excreted in urine expressed as µg excreted/h. Shaded area shows µg α-zearalenol excreted/h. Volume of urine (- - - - -) collected expressed as mL urine/h.

Sample Collection

One female pig of Swedish Landrace × Yorkshire breed, weighing 65.4 kg, was fed 1.03 kg standard diet A (10) containing 5 mg zearalenone/kg diet. Blood samples were taken frequently (Figure 1) from a permanent blood cannula, which was introduced into the carotid artery (11), and urine was collected continuously, until 8 h after feeding, via ballon catheter fitted in the urinary bladder. Blood samples were collected in heparinized tubes and centrifuged (3000 g, 20 min), and plasma was removed with a Pasteur pipet. Plasma and urine were kept frozen (−20°C) until analysis.

Preparation of Spiked Blood Plasma and Urine Samples

Prepare standard solutions of zearalenone (200 ng/mL methanol) and α- and β-zearalenol (200 and 500 ng/mL methanol, respectively). Add 0–750 µL portions of standard solution to separate 15 mL glass tubes and evaporate to dryness under nitrogen and gentle heat (50°C). Transfer 5.0 mL blank plasma or urine to tube and redissolve standard solution by vortex-mixing. Proceed with incubation and cleanup procedures described below.

Incubation

Transfer 5 mL spiked or unspiked plasma or urine to 15 mL glass tube, add 5 mL 0.2M sodium acetate (pH 5.5) and 50 µL β-glucuronidase plus sulphatase solution (type H-2). Incubate 16 h at 37°C. Stop incubation by adding 1 mL 3M H_3PO_4.

Sample Cleanup

After incubation, extract sample twice with 25 mL chloroform, or pour sample onto extraction column and extract with 40 mL chloroform into 100 mL round-bottom flask. Evaporate to dryness on rotary evaporator. Dissolve residue in 1 mL toluene and load onto silica cartridge. Wash flask with 10 mL toluene and wash silica cartridge with same solution. Elute zearalenone and zearalenols with 10 mL toluene–acetone (88 + 12) into 50 mL round-bottom flask and evaporate to dryness. Transfer residue in 2 mL chloroform into 15 mL conical glass tube. Extract by adding 2 mL 0.18M NaOH and vortex-mix thoroughly for three 10 s periods. Centrifuge at 4000 × *g* for 1 min and remove upper NaOH layer with Pasteur pipet into fresh 15 mL conical tube. Neutralize basic solution with 0.5 mL 0.4M H_3PO_4 and re-extract twice with 2 mL chloroform by vortex-mixing each time for two 15 s periods and centrifuging as above. Discard upper aqueous layer and combine chloroform extracts. Remove any residues of aqueous layer by adding very small amounts of anhydrous sodium sulfate, ca 150 mg, and vortex-mix 15 s. Repeat, if any aqueous droplets are left. Centrifuge as above and transfer chloroform extract quantitatively into 1.5 mL (plasma) or 2.5 mL (urine) glass tube by evaporating to dryness under nitrogen and gentle heat (50°C). Immediately before injection onto LC column, dissolve plasma and urine residues in 50–100 µL and 500–1000 µL, respectively, LC mobile solvent by vortex-mixing 15 s. Inject 10 µL sample onto LC column.

Determination

Identify zearalenone and α-zearalenol peaks by comparing retention times with those of standards. Measure peak areas geometrically, and determine zearalenone and α-zearalenol contents graphically from comparable standard curve of zearalenone and α-zearalenol.

Confirmation.—Confirm identity sample peak height ratios by sequential LC analyses according to Ware and Thorpe (12).

Results and Discussion

When elaborating a method for the detection of zearalenone and its metabolites, one needs to consider both free and conjugated forms of the toxin. Previous work by Mirocha et al. (5) showed that administration of 90 mg zearalenone/kg body weight to an 8 kg piglet resulted in 31% of total urinary zearalenone in the glucuronide form. No sulfate form of the metabolites could be detected.

In our investigation, when giving a very small amount of zearalenone to a 65 kg pig, corresponding to 80 µg/kg body weight, we found in urine both conjugated zearalenone and α-zearalenol and only trace amounts of free substances (0.4 ng/mL). In plasma, all zearalenone and α-zearalenol were found conjugated. The amount of free substances in urine and plasma was measured by excluding the incubation with β-glucuronidase or sulfatase. Zearalenone and α-zearalenol in urine were found to be conjugated with glucuronic acid based on the results of an experiment in which urine was collected from a pig 7 h after it was fed with zearalenone-contaminated feed (5 mg/kg). Before incubation with β-glucuronidase or sulfatase plus β-glucuronidase, urine was precipitated with saturated $BaCl_2$ solution to remove any interfering sulfate ions, but the addition of sulfatase to the incubation did not release any more bound zearalenone or α-zearalenol. The mean values of 5 determinations each of free zearalenone and free α-zearalenol after incubation with β-glucuronidase (5000 u) were 44.4 ± 4.8 and 68.8 ± 6.7 ng/

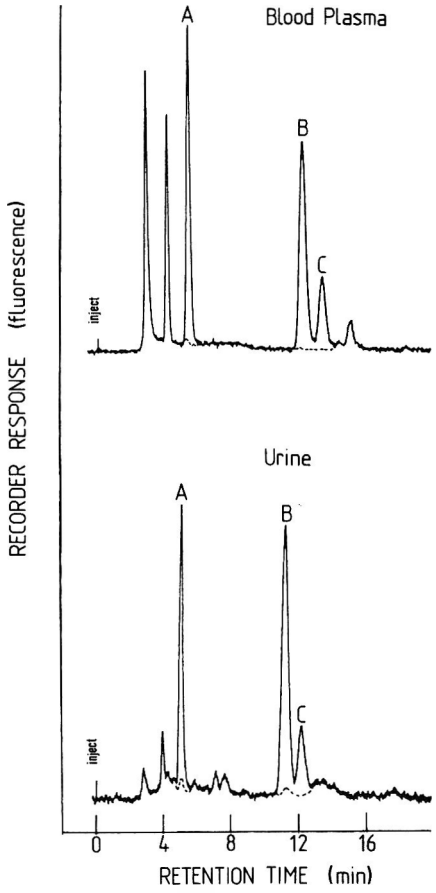

Figure 2. LC chromatograms of (A) zearalenone, 2.4 ng in both plasma and urine; (B) α-zearalenol, 2.6 ng in plasma and 3.0 ng in urine; (C) β-zearalenol, 6.0 ng in both plasma and urine. Injected red samples, 1.0 mL plasma and 0.05 mL urine. Dotted lines denote response of blanks. See text for LC conditions.

mL, and after incubation with β-glucuronidase (5000 u) plus sulfatase (255 u) were 40.4 ± 1.8 and 61.2 ± 1.8 ng/mL, respectively. This finding is consistent with literature reports on the conjugation mechanism (13), which state that pigs excrete phenol bound 100% as glucuronides. Because of large species variations for glucuronide and sulfate conjugation, we used a mixture of β-glucuronidase and sulfatase in our incubations. This renders the method suitable for species other than swine. Preliminary work showed that at least 16 h of incubation at 37°C was necessary to release all bound substances. The incubations not only liberated bound zearalenone and α-zearalenol, but also numerous contaminants. After cleanup on silica cartridge and acid-base extraction, the interference of other substances was removed as shown in the LC chromatograms (Figure 2).

Retention times for zearalenone and α-zearalenol were about 5 min and 11 min, respectively. Preparing a new mobile solvent for LC caused retention times to vary up to 0.4 min between mobile phases, which necessitated always making standard injections. However, these variations did not affect resolution of the peaks. The chromatograms (Figure 1) showed that both α- and β-zearalenol could be detected by our method and that the peaks were resolved from one another. The fluorescence of β-zearalenol was weaker than that of zearalenone and α-zearalenol, which made the method less sensitive for β-zearalenol. The detection limits of zearalenone and α-zearalenol were 0.5 ng/mL plasma and 5 ng/mL urine. The detection limit of β-zearalenol was about 4 times higher.

Smaller amounts of zearalenone or zearalenols could be easily detected by increasing the LC injection volume or the initial sample volume.

Figure 3 shows a plot of the fluorescence detector response vs amounts of standard zearalenone and α-zearalenol injected in the range 0–8 ng. The response was linear over the whole range, and the correlations, area to amount, were 0.997 for zearalenone and 0.998 for α-zearalenol.

Recovery results (Tables 1 and 2) and fluorescence detector response (Figure 3) of zearalenone and zearalenols showed that the method was quantitative. There were no differences in recoveries between the extraction procedures, but using the extraction column was faster.

When detecting such low levels of zearalenone and zearalenol as described in this paper, contamination of the sample with zearalenone originating from the LC injector can occur. This can happen, for instance, when the LC apparatus has previously been used with a more concentrated standard solution. The problem was solved, in our case, by rinsing the injector with methanol and LC mobile solvent and changing the PTFE plug (Waters, No. 96039), which in time became scratched.

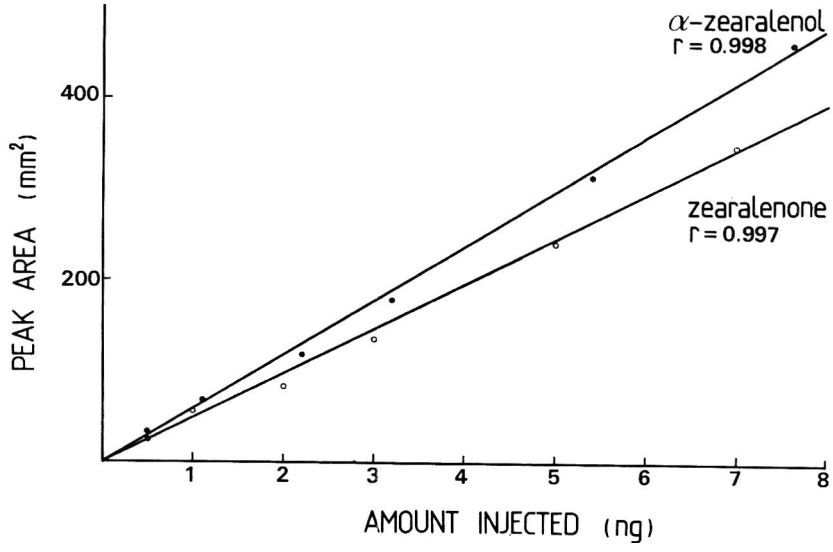

Figure 3. Plot of fluorescence detector response vs amount of zearalenone and α-zearalenol standard injected onto LC column; r = correlation coefficient.

Table 1. Recoveries of zearalenone and α- and β-zearalenol from spiked blood plasma incubated with β-glucuronidase + sulphatase for 16 h

Compound added, ng/mL	No. of analyses	Rec., % ± SD
Zearalenone		
2.0	3	83 ± 7.6
10.0	3	83 ± 0.6
α-Zearalenol		
2.2	3	89 ± 2.9
10.8	3	80 ± 1.0
β-Zearalenol		
5.0	3	81 ± 6.4
24.8	3	84 ± 1.1

Table 2. Recoveries of zearalenone and α- and β-zearalenol from spiked urine unincubated* or incubated with β-glucuronidase + sulphatase for 16 h

Compound added, ng/mL	No. of analyses	Rec., % ± SD
Zearalenone		
4.0*	3	78 ± 2.6
10.0	3	90 ± 8.1
30.0	6	88 ± 7.6
α-Zearalenol		
4.3*	3	72 ± 2.2
10.0	3	81 ± 3.0
32.0	6	82 ± 8.5
β-Zearalenol		
10.0*	3	84 ± 2.7
127.0	3	82 ± 6.4

The feeding experiment (Figure 1) showed that zearalenone was rapidly metabolized into α-zearalenol. As early as 30 min after feeding, 2.0 ng/mL of zearalenone and 3.2 ng/mL of α-zearalenol could be detected in the blood plasma. Six samples examined during 8 h after feeding showed a plasma content of α-zearalenol 1.5 to 6 times as high as that of zearalenone. The maximum amount of zearalenone and α-zearalenol together (8.3 ng/mL) in plasma occurred 4.5 h after feeding. In urine, the maximum amount was collected between 3.5 and 5 h after feeding.

No β-zearalenol could be detected in plasma or urine. Mirocha et al. (5) found 5% of total metabolites in pig urine to be β-zearalenol when they administered a 1000-fold higher amount of zearalenone than that used in this investigation. This 5% concentration would, at maximum excretion, correspond to 18 ng β-zearalenol/mL urine, which is less than the detection limit.

A striking difference between plasma and urine was the ratio of zearalenone to zearalenol. The urine excretion of α-zearalenol was consistently lower than that of zearalenone until 6 h after feeding, when α-zearalenol reached its maximum excretion level. After 6 h, the urine contained more α-zearalenol than zearalenone. The total amount of zearalenone and α-zearalenol excreted in urine during 8 h after feeding corresponded to 15.6% of the total ingested zearalenone.

The maximum amounts of zearalenone and α-zearalenol in urine corresponded to 216 ng/mL and 153 ng/mL urine, respectively. Compared with the maximum level in plasma, these amounts are very easily detected. In other words, urine can be very suitable for the detection of zearalenone and its metabolites in animals suspected of ingesting contaminated feed.

The method described here was rapid and sensitive for the determination of both free and conjugated zearalenone and its metabolites in blood plasma and urine. Pharmacokinetic studies of zearalenone metabolism in vivo can be made by using the analytical method described, which will be used in subsequent investigations at our laboratory.

Acknowledgments

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Negative Ion Chemical Ionization Mass Spectrometric Method for Confirmation of Identity of Aflatoxin B_1: Collaborative Study

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An interlaboratory study of a negative ion chemical ionization mass spectrometric (MS) confirmation procedure for aflatoxin B_1 was conducted in laboratories in the United States, England, and West Germany. Twelve partially purified, dry film extracts from naturally and artificially contaminated roasted peanuts, cottonseed, and ginger root containing varying quantities of aflatoxin B_1 were distributed to the participating laboratories. The extracts required additional cleanup before MS analysis, using either an acidic alumina column and preparative thin layer chromatography (TLC) or a 2-dimensional TLC procedure. Recovery of purified aflatoxin B_1 was influenced by the degree of recovery of sample from acid alumina and/or the TLC plate and incomplete elution of aflatoxin B_1 from silica gel. Factors affecting MS confirmation included the purity and recovery of aflatoxin and MS instrument sensitivity. Aflatoxin B_1 identity was confirmed in 19.5, 90.9, and 100% of samples containing <5, 5–10, and >10 ng aflatoxin B_1/g product, respectively, by solid probe introduction using full mass scans. The MS method has been adopted official first action.

Mycotoxins, toxic compounds produced by molds, may be present in foods and feeds. Ingestion of mycotoxin-contaminated products can cause pathological responses in animals and humans. Aflatoxins, which are produced by the ubiquitous molds *Aspergillus flavus* and *A. parasiticus*, are the most significant mycotoxins on the basis of concern engendered and research stimulated. The most potent of the aflatoxins, aflatoxin B_1, is a hepatocarcinogen and is toxic in many animal species (1).

A great deal of research has been directed to the development of analytical methods for the determination of low levels of aflatoxins in foods and feeds and for the confirmation of the identity of aflatoxin B_1 (2). Both liquid and thin layer chromatography (TLC) have been used in the determinative step with good precision. However, the procedures for the confirmation of aflatoxin B_1, based on either physical or chemical properties or biological activity, are time consuming, and in some cases nonspecific (3). To achieve high specificity in confirmation of aflatoxin identity, various mass spectrometric (MS) techniques have been investigated, including low resolution electron ionization (4), high resolution multiple ion monitoring with solid probe sample introduction (5), on-column gas chromatography/high resolution MS with ion monitoring (6), and MS/MS using solid probe sample introduction (R. D. Plattner, U.S. Department of Agriculture, Peoria, IL, personal communication, 1983).

The purpose of this study was to evaluate the applicability of the negative ion chemical ionization (NICI) technique to confirm the identity of aflatoxin B_1 in roasted peanuts, cottonseed, and ginger root by using previously developed analytical methods (7, secs **26.026–26.030, 26.052–26.056;** 8) and selected isolation procedures (3; 7, secs **26.030, 26.076–26.079;** 9).

Collaborative Study

Nine chemical laboratories were each furnished 12 coded, partially purified extracts containing aflatoxin B_1, a description of the final cleanup method, and a report form. Each dry film extract was equivalent to a 10 g sample of roasted peanuts, cottonseed, or ginger root naturally and artificially contaminated with aflatoxin B_1. The extracts were prepared by methods applicable to the respective types of samples: **26.029** for peanut butter (7), **26.055–26.056** for cottonseed (7), and a special method for ginger root (8). Contamination concentrations ranged from nondetectable levels to 50 ng aflatoxin B_1/g. The purified extracts were then given to 6 MS laboratories for confirmation of identity of aflatoxin B_1. Collaborators were also supplied with a practice sample at a concentration of 20 ng/g to become familiar with the cleanup and MS confirmation methods. Reference standards with directions for dilution to working concentrations were provided.

Collaborators were instructed to use either *(1)* acid alumina column chromatographic cleanup or *(2)* 2-dimensional TLC cleanup on the extracts and to quantitate the recovered aflatoxin B_1 before MS analysis (Figure 1). They were instructed to quantitatively transfer the entire sample extract to the column or TLC plate as applicable, using standard laboratory procedures (7).

Aflatoxin B_1 Identification

Confirmation Method

First Action

(Level of confirmation of identity is >5 ng aflatoxin B_1/g.)

26.A01 ***Principle***

Partially cleaned up B_1 exts are further cleaned up by 2-dimensional thin layer chromatgy. Presence of aflatoxin B_1 is confirmed by neg. ion chemical ionization mass spectrometry (MS).

26.A02 ***Apparatus***

(a) *Thin layer plates.*—20 × 20 cm with 0.25 mm silica gel and silica gel 60 precoated (E. Merck), or equiv.

(b) *Development tank.*—Glass tank with ground-to-fit cover and metal trough.

(c) *Thin layer plate scraper.*—Kontes K416400-0022, or equiv.

(d) *Glass fiber filter paper.*—4.25 cm, Whatman GF/A, or equiv.

(e) *Membrane filter.*—0.5 μm PTFE, Millex-SR filter unit (Millipore Corp. SLS RO25NS), or equiv. filter to male Luer outlet of 5 or 10 mL syringe.

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[2]Division of Chemical Technology.

[3]New York Regional Laboratory, FDA, Brooklyn, NY 11232.

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The recommendation of the Associate Referee was approved by the General Referee and Committee C and was adopted by the Association. See the General Referee and Committee reports, *J. Assoc. Off. Anal. Chem.* (1985) **68**, March issue.

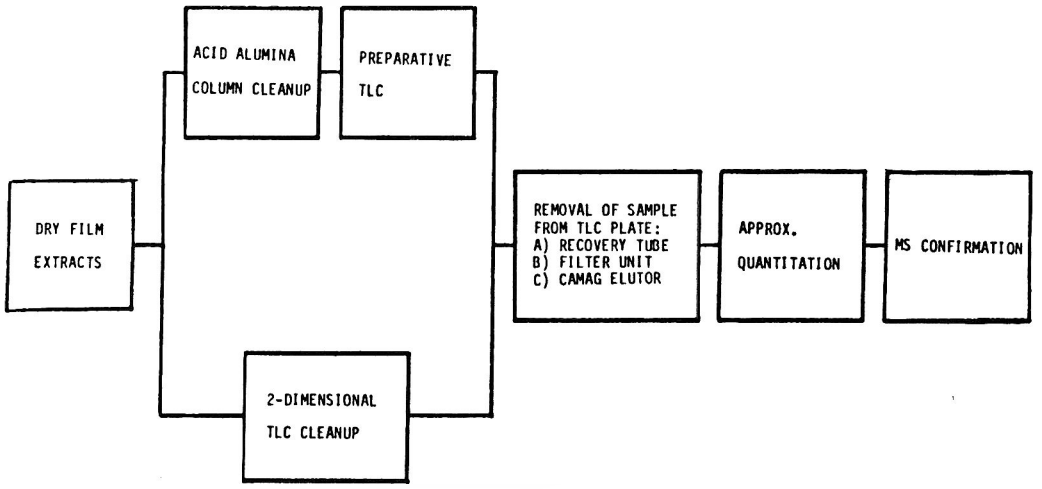

Figure 1. Sample analysis scheme.

(f) *Automatic TLC spot eluter.*—Camag Eluchrome 75000, or equiv.

(g) *Concentrator tube.*—2 mL, Kontes K422560, or equiv.

(h) *Mass spectrometer.*—Finnigan MAT 3300 interfaced to INCOS 2300 data system with following operating conditions: 55–655 Daltons scan range, 3.0 s scan time, 0.7 torr source pressure, 120° source temp., CH_4 reagent gas, 0.50 mA filament current, and 140 eV electron energy, or equiv.

26.A03 *Reagents*

(a) *Solvents.*—ACS grade: acetone, benzene, CH_3CN, $CHCl_3$, anhyd. ethyl ether, isopropanol, and MeOH. Caution: Do not use any solvs stored in plastic ware because leachables may interfere in interpretation of mass spectra.

(b) *Mass spectrometer calibrants.*—Fomblin (PCR, Gainesville, FL), or perfluorotributylamine (FC-43) (PCR) spiked with dinitrofluorobenzene, (Aldrich Chemical Co.), and trifluoroacetic anhydride (PCR).

(c) *Mixed aflatoxin reference std.*—B_1 and G_1, 0.5 μg/mL; B_2 and G_2, 0.15 μg/mL in benzene–CH_3CN (98 + 2).

(d) *Aflatoxin B_1 reference std.*—10 μg/mL in acetone.

26.A04 *Preparation of Extracts*

Prep. ext of roasted peanuts as in **26.026–26.030**, cottonseed as in **26.052–26.056**, and ginger root as in *JAOAC* **63**, 1052(1980).

26.A05 *Chromatographic Cleanup*

Perform addnl cleanup using 2-dimensional TLC.

Take thin layer plates and score as shown in Fig. **26:A1**. Spot 5 μL mixed aflatoxin ref. std for development in each direction. Dissolve unknown sample in 50 μL $CHCl_3$ and spot 2 μL ca 1 cm apart from each std spot. Apply remaining sample as single spot in corner of TLC plate. Rinse sample vial with 20 μL $CHCl_3$ and superimpose rinse on sample spot. Develop plate in 2 dimensions, using following solvs: first, acetone–$CHCl_3$ (1 + 9) and second, anhyd. ethyl ether–MeOH–H_2O (96 + 3 + 1). Let plate stand 10 min in dark between developments to remove solv. After second development, let plate stand in hood 5 min to evap. developing solv. Locate aflatoxins under longwave UV light, mark, and elute from TLC plate as described below. (Caution: Aflatoxin recovery will be reduced with prolonged exposure to UV and fluorescent light.)

26.A06 *Elution of Purified Aflatoxin B_1*

Elute the sample from 2-dimensional TLC plate by procedure **a**, **b**, or **c**:

(a) *TLC vacuum scraper.*—Proceed after development of TLC plate as described in **26.079**, using TLC scraper **26.076(b)** or **26.A02(c)**. Use six 1 mL portions of $CHCl_3$ to elute aflatoxin from the silica gel into 2 dram vial.

(b) *Scrape and filter.*—Mark aflatoxin spot as in **26.079**. Scrape spot onto creased, 10 × 10 cm piece of Al foil and transfer to glass fiber filter paper, (**d**), placed in 25 mm funnel (1 cm stem). Wash Al foil and elute aflatoxin B_1 into 2 dram vial with six 0.5 mL portions of $CHCl_3$. (TLC spot may also be scraped onto weighing paper and transferred dry onto filter without washing the paper.) Alternatively, filter scraped silica gel as described, using glass syringe filter, (**e**). Wash aflatoxin B_1 into 2 dram vial with six 0.5 mL portions of $CHCl_3$.

(c) *In situ elution.*—Elute aflatoxin B_1 spot with 2.5 mL $CHCl_3$–acetone–isopropanol (85 + 15 + 5) into concentrator tube, (**g**).

Evap. purified aflatoxin soln prepd in (**a**), (**b**), or (**c**) to dryness under N stream in warm H_2O bath (50°). Transfer to 2 mL concentrator tube, (**g**), if sample is not already in one, and evap. to dryness

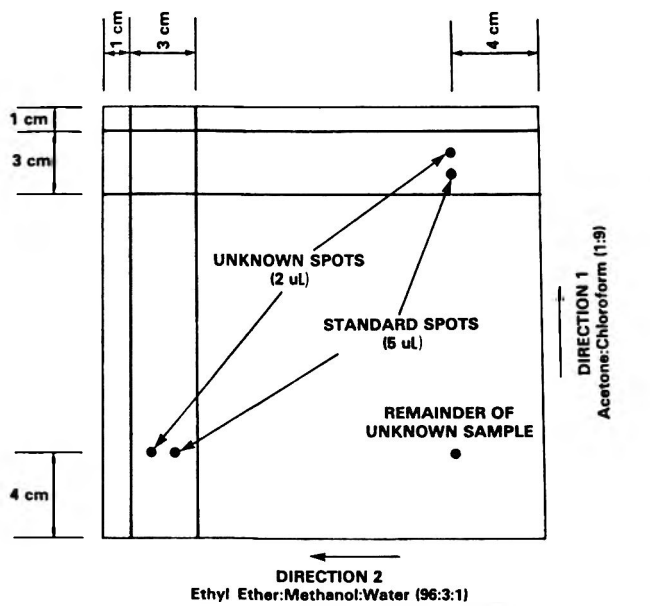

FIG. 26:A1—Spotting and scoring patterns for 2-dimensional TLC plate

as described above. Save for mass spectrometry. If necessary, store as dry film in freezer.

26.A07 *Quantitation of Aflatoxin B_1*

Analyze by TLC as in **26.031**. Dissolve purified aflatoxin B_1 in 50 μL benzene–CH_3CN (98 + 2) and spot 5 μL on silica gel TLC plate. Also spot 5 and 10 μL mixed aflatoxin ref std. Develop with acetone–$CHCl_3$ (1 + 9). R_f of aflatoxin B_1 should be ca 0.5 (see **26.008–26.013**). Evap. remaining ext (45 μL) to dryness under stream of N and save for mass spectrometry. If necessary, store as dry film in freezer.

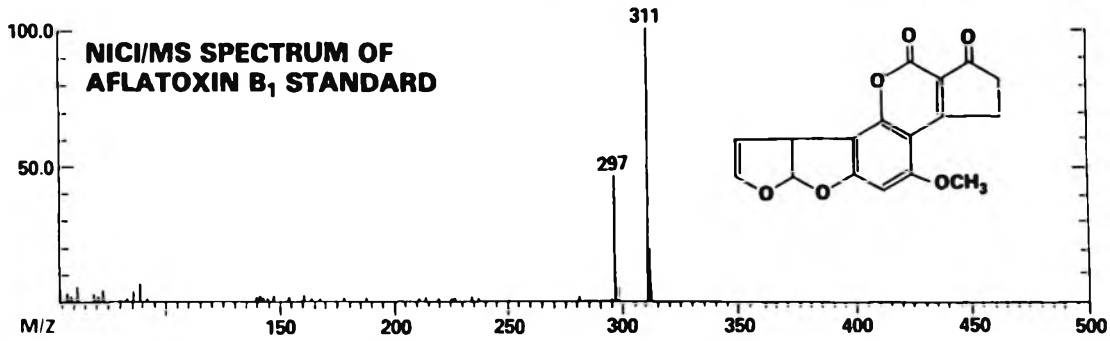

FIG. 26:A2—NICI/MS spectrum of aflatoxin B_1 standard

Table 1. Aflatoxin B_1 found (%) after final cleanup of partially purified extracts of roasted peanuts, cottonseed, and ginger root by column chromatography or 2-dimensional (2-D) TLC

Sample	Aflatoxin B_1 contam., ng/g	Cleanup method[a]	
		Chromatogr.	2-D TLC
Roasted peanuts	ND[b]–10	33 (6)	30 (10)
	11–25	168 (3)	76 (5)
	>25	259 (2)	74 (5)
Cottonseed	ND–10	ND (2)	ND (5)
	11–25	2 (2)	39 (5)
	>25	57 (6)	76 (10)
Ginger root	ND–10	92 (5)	32 (10)
	11–25	167 (3)	41 (5)
	>25	139 (3)	77 (5)

[a]Number of samples in parentheses.
[b]Not detected.

26.A08 ***Mass Spectrometry***

(a) *Mass spectrometry of std aflatoxin B_1.*—Transfer up to 10 μL (100 ng) B_1 ref. std to MS direct inlet probe glass holder.

Analyze samples in neg. ion mode. Calibrate mass scale from m/z 100 to 500, using appropriate calibrant, such as Fomblin, or FC-43 (spiked with dinitrofluorobenzene and trifluoroacetic anhydride to provide ref. peaks in lower mass range). Optimize source tuning and reagent gas pressure with calibrant. Adjust instrument parameters to obtain good quality spectrum from 10 ng aflatoxin B_1 by direct probe introduction. (Note: Source temp. has pronounced effect on spectrum and must be adjusted and maintained as const as possible during course of analysis.) Obtain spectrum of aflatoxin B_1 ref. std (Fig. **26:A2**) and repeat 3 times under identical conditions to det. instrument reproducibility. Major peaks are m/z 297, 311, and 312. Interference peaks should be less than 20%. Spectra without ion at m/z 311 have been produced on some instruments.

(b) *Mass spectrometry of sample.*—Dissolve purified sample ext in acetone to give 10 ng B_1/μL. Add small vol. of acetone to clean sample vial, agitate, and evap. to ca 10 μL. Transfer quant. to inlet probe and run to test for ghost peaks. Transfer 10 μL sample soln (100 ng B_1) to inlet probe for MS analysis. Repeat analysis of aflatoxin B_1 ref. std to verify instrument performance. Confirm presence of aflatoxin B_1 in sample by comparison of spectrum of sample to spectrum of std. Examples are shown in Fig. **26:A2**.

Results and Discussion

MS confirmation procedures usually require purity beyond the normal cleanup used for chemical confirmation methods. The final cleanup procedure, at the option of the analyst, must meet certain criteria, including removal or separation of interfering substances and sufficient recovery of the sample to allow confirmation of identity. Five collaborators used the 2-dimensional technique suggested by the authors. With the exception of some difficulty with fluorescence tailing, this procedure proved to be the most successful. One collaborator reversed the order of developing solvents and obtained excellent results. Another collaborator used silica on aluminum TLC plates, cut out the area containing the purified toxin, eluted with chloroform–methanol (1 + 1), filtered over a sea sand minicolumn, and obtained excellent results.

Purification of aflatoxin B_1 by chromatography on acid or neutral alumina in the authors' laboratories usually produces recoveries between 80 and 90%. However, in the collaborative study, none of the 3 collaborators trying this technique was successful. Two reported 0–44% recoveries and one >200%. One collaborator was given a second set of samples and continued the study using the 2-dimensional cleanup technique. Another collaborator substituted silica gel (Merck 60) and reported recoveries ranging from 5 to 38%. Therefore, these collaborators were unable to successfully confirm the aflatoxin identity in many of the samples. In investigating the problem during the study, we found that the most likely cause was variability in the commercially available alumina. It must, therefore, be emphasized that regardless of the cleanup procedure used, its performance must be checked with standards and known samples.

Several techniques were used successfully for removing aflatoxin B_1 from the TLC plate. Six analysts scraped the silica gel from the TLC plate and eluted the aflatoxin B_1 by either filtering the silica gel with glass fiber paper or a Millipore filtering unit. One collaborator used a vacuum plate scraper and another used the aluminum-backed TLC plates previously described. Some collaborators substituted chloroform–methanol (1 + 1) and (97 + 3) for chloroform to elute

Table 2. Collaborative results for aflatoxin B_1 confirmation of identity by NICI/MS

Sample	Aflatoxin B_1, ng/g									
	<5		5–10		11–20		21–30		>30	
	+	–	+	–	+	–	+	–	+	–
Roasted peanuts	3	10	4	1	2	0	1	0	1	0
Cottonseed	3	11	2	0	2	0	1	0	3	0
Ginger root	2	12	4	0	2	0	2	0	1	0
Aflatoxin B_1 identity confirmed, % of samples	19.5		90.9		100		100		100	

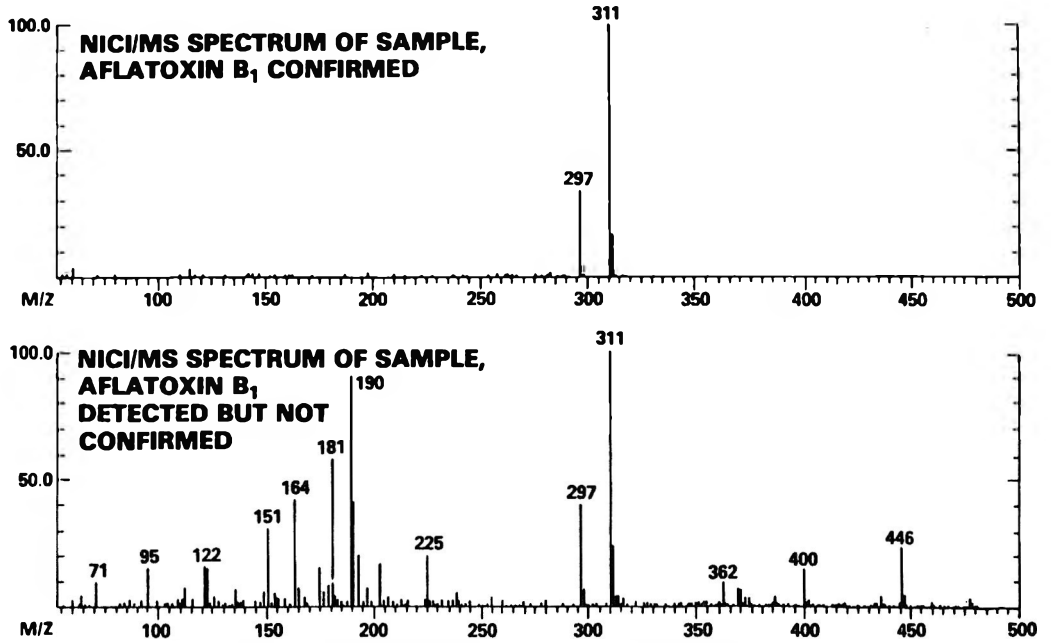

Figure 2. Mass spectra of samples containing aflatoxin B_1, showing effect of interfering substances on confirmation of identity of aflatoxin B_1.

the B_1 from the silica gel. One collaborator observed that chloroform stabilized with a nonpolar hydrocarbon will not elute all the aflatoxin B_1 and suggested increasing the polarity of the chloroform by adding methanol to improve recoveries. Table 1 shows the recovery of aflatoxin B_1 obtained by the collaborators based on concentrations in the original samples. The 2-dimensional TLC technique showed the most consistent results.

Six laboratories conducted the NICI/MS analyses, and the results are presented in Table 2. These results show a limit of confirmation of identity around 5 ng/g. Aflatoxin B_1 identity was confirmed in 19.5, 90.9, and 100% of the samples containing <5, 5–10 ng aflatoxin B_1/g product, respectively, by solid probe introduction using full mass scans. It was necessary to introduce about 50 ng aflatoxin B_1 into the MS instrument to override interfering substances remaining in the sample extract. The spectra in Figure 2 show the effect of interfering substances on the quality of the spectra. Although peaks at m/z 297, 311, and 312 are present in relative intensities approximating the standard, they could also be daughter ions of the higher mass compounds. The major factors affecting MS confirmation were purity of the isolated substance, recovery of the purified substance from the column and/or TLC plate, and poor instrument sensitivity. Obviously, with good recoveries of a highly purified aflatoxin B_1 and using a well-tuned instrument, the limit of confirmation of identity could be lowered significantly. Figure 3 shows the relationship between recovery and concentration of the toxin with respect to confirmation. For samples where aflatoxin B_1 was detected but not confirmed (Figure 2), the sample should be subjected to an additional cleanup process and reanalyzed by MS to confirm the identity of the toxin.

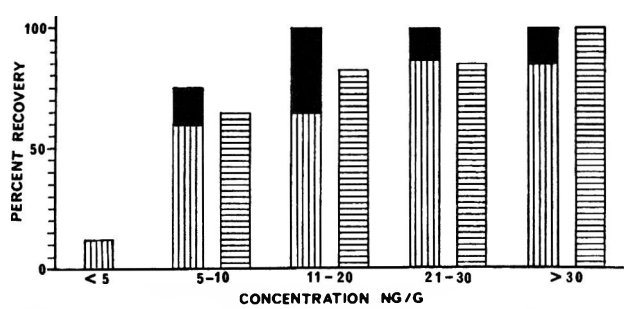

Figure 3. Comparison of aflatoxin B_1 identity confirmation with recovery of original contamination. Vertical bars = B_1 confirmed; solid bars = B_1 detected but not confirmed; horizontal bars = % recovery.

Recommendation

It is recommended that the NICI/MS method for confirmation of identity of aflatoxin B_1 be adopted official first action.

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Liquid Chromatographic Determination of Tenuazonic Acid and Alternariol Methyl Ether in Tomatoes and Tomato Products

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A liquid chromatographic (LC) method for determining tenuazonic acid (TA) and alternariol methyl ether (AME) in tomatoes and tomato products is described. The *Alternaria* metabolites are extracted from a water slurry of the sample with $CHCl_3$, the mixture is centrifuged, and the extract is fractionated on a silica gel column. Reverse phase LC with an ultraviolet detector (for TA) and a fluorescence detector (for AME) connected in series is used for final separation and determination. The limit of determination for TA and AME is 25 and 3 ng/g, respectively, with average recoveries from catsup of 83 and 68%, respectively. The LC method also detects alternariol, but interfering peaks in some samples prevent accurate quantitation. Chemical ionization mass spectrometry (CI MS) is used to confirm TA. Samples (142) of tomatoes collected from commercial processing lines were analyzed; TA was found in 73 samples (0.4–70 μg/g).

One of the most common groups of molds that contaminate foods and feeds is the genus *Alternaria*. *Alternaria* are indigenous to soil, and consequently species of *Alternaria* are frequently found growing on crops before harvest (1). In a recent survey of small grains in the United States, species of *Alternaria* were found in 184 of 230 samples (2). In addition, *Alternaria* grow at low temperatures and consequently are frequently found on fruits and vegetables subjected to cold stress. Rotting of fruits and vegetables caused by *Alternaria* results in considerable economic losses annually to the food industry (3). In a recent survey of 146 tomato samples, *Alternaria* was the predominant genus present on eastern/midwestern products (P. Mislivec, unpublished data, 1984).

Alternaria produce many metabolites of diverse chemical structure (4–6), some of which exhibit mammalian toxicity (7, 8). One of these metabolites, tenuazonic acid (TA), has been implicated in onyalai, a common hematologic disorder of humans in certain areas of Africa (9). Another metabolite, alternariol methyl ether (AME) is toxic to both the dam and fetus of Syrian golden hamsters, and is cytotoxic and mutagenic (4). At the present time, little information is available to evaluate the toxicological importance of human exposure to the *Alternaria* metabolites, because both knowledge of exposure to the metabolites and information on their toxicological effects are lacking. To provide information on human exposure, several analytical procedures, including thin layer chromatography (10, 11) and liquid chromatography (LC) (12–18), have been developed for analysis of foods and feeds for the known major metabolites: AME, TA, alternariol (AOH), and altenuene. These methods have been used in limited surveys of commodities to detect the presence of the *Alternaria* metabolites (11, 14, 17).

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Recently there has been interest in developing a chemical method for controlling the quality of a processed food by analyzing the raw materials used in the preparation of such foods for mold metabolites (19). We have considered one aspect of this problem: detecting the use of *Alternaria*-contaminated tomatoes in the preparation of catsup by detection of the *Alternaria* metabolites in the catsup. A good analytical method for analysis of tomatoes and tomato products was needed. This paper describes the LC method that was developed.

METHOD

Apparatus

(a) *Explosion-proof blender.*—Waring Model EP-1 with 1 L jar and cover.

(b) *Centrifuge.*—Sorvall Model RC-2B with rotor for 250 mL polyethylene bottles (Du Pont Instruments, Wilmington, DE 19898).

(c) *Chromatographic tubes.*—22 × 300 mm with Teflon stopcock, 250 mL reservoir type.

(d) *Liquid chromatograph.*—Waters Model 6000A pump, U6K injector, Z module radial compression system with C18 column to fit, Model 440 absorbance detector, and Model M730 data module (Waters Associates, Milford, MA 01757); Kratos Model FS 970 fluorescence detector (Kratos, Westwood, NJ 07675).

(e) *Mass spectrometer.*—Finnigan 3300 chemical ionization (CI) system with INCOS 2300 data system (Finnigan MAT, San Jose, CA 95134). Electronics modified to incorporate dual detectors and ion source pulsed at 10 kHz as described in Ref. 20.

Reagents

(a) *LC mobile phase.*—Methanol–water (85 + 15) with 300 mg $ZnSO_4.7H_2O$/L.

(b) *Standards.*—1 μg each of TA, AME, and AOH/mL methanol. TA, AME, and AOH are available from the senior author.

TA is stored as the copper salt. Reconvert weighed amount (2 mg) by passing through Dowex 50W-X8 minicolumn as described in Ref. 14, except use methanol instead of methylene chloride for the solvent. Measure UV spectrum to determine concentration of ca 25 μg/mL solution. Absorptivity is 13 000; mol. wt is 197. Dilute to 1 μg/mL methanol. Prepare fresh standard every week because TA isomerizes on standing in solution.

(c) *Silica gel for column chromatography.*—E. Merck silica gel 60, 0.063–0.2 mm.

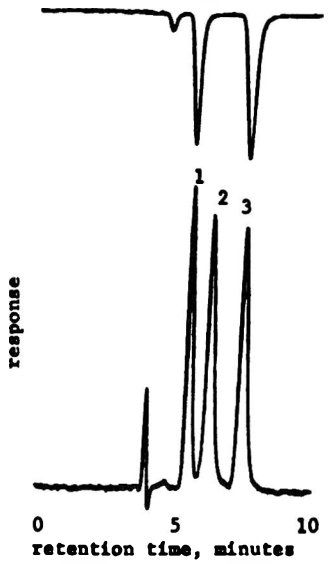

Figure 1. LC of 1, alternariol; 2, tenuazonic acid; and 3, alternariol methyl ether. Upper, fluorescence detector trace; lower, 280 nm absorbance detector trace.

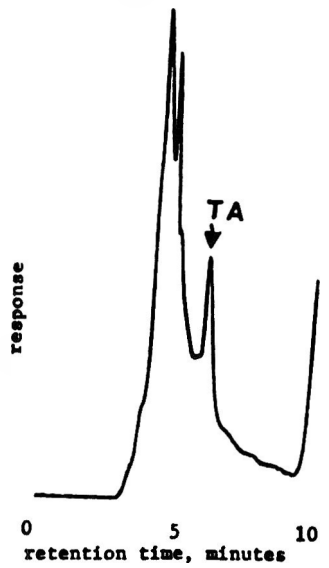

Figure 2. LC of extract of tomato containing tenuazonic acid (TA), 1.4 μg/g.

(d) *Methane.*—99.99% purity (Matheson Gas Products, E Rutherford, NJ 07073).

Extraction

Place 50 g sample in blender jar. Add 450 mL water and 100 mL $CHCl_3$, and blend 3 min at high speed. Decant into two 250 mL centrifuge bottles. Centrifuge 10 min at 5000 rpm. Decant liquid into 500 mL separatory funnel. Collect 50 mL lower phase in 50 mL graduated cylinder. At this point 10 μL $CHCl_3$ solution of extract can be injected into liquid chromatograph or, if greater sensitivity is desired, the extract may be cleaned up by column chromatography.

Column Chromatography

Place ball of glass wool loosely in bottom of 22 × 300 mm chromatographic tube and add $CHCl_3$ until tube is ca ½ full; then add 25 mL silica gel. Wash sides of tube with ca 20 mL $CHCl_3$ and stir to disperse silica gel. When rate of settling slows, drain some $CHCl_3$ to aid settling, leaving 5–7 cm above silica gel. Slowly add 10 mL anhydrous Na_2SO_4. Add 50 mL sample extract to column and elute at maximum flow rate; when level of extract reaches top of Na_2SO_4 layer, add 50 mL $CHCl_3$. When level of $CHCl_3$ reaches top of Na_2SO_4 layer, change receiver and add 50 mL $CHCl_3$–methanol (9 + 1). When column stops dripping, change receiver and add 50 mL methanol containing 300 mg $ZnSO_4.7H_2O$/L to column. Evaporate each of the 3 fractions to ca 5 mL on steam bath and transfer to separate 8 mL vials. Evaporate to dryness on steam bath under stream of nitrogen. Dissolve in 1 mL methanol.

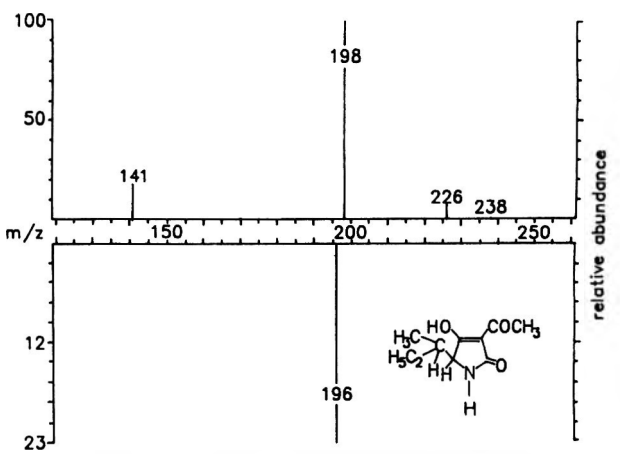

Figure 3. Positive ion (top) and negative ion (bottom) CI mass spectra of tenuazonic acid isolated from tomato sample. Methane source pressure 1 torr, emission current 0.5 mA at 140 eV, source temperature 140°C, positive ion multiplier sensitivity set at 10× negative ion multiplier sensitivity.

Liquid Chromatography

Equilibrate column ≥1 h with mobile phase. Set absorbance detector at 280 nm, 0.005 absorbance unit full scale. Set fluorescence detector excitation wavelength at 278 nm and insert 370 and 389 nm emission cutoff filters. Set sensitivity so that injection of 10 ng AOH gives ca full-scale response. Inject 10 μL combined standard. AOH should elute in 5.6 min, TA in 6.3 min, and AME in 7.5 min. The water–methanol ratio of the mobile phase may need to be slightly altered to obtain baseline resolution of the 3 standard peaks. Inject 10 μL extract. Separately inject the 3 fractions from each sample. AME should be in the $CHCl_3$ fraction, AOH in the $CHCl_3$–methanol (9 + 1) fraction, and TA in the methanol–$ZnSO_4$ fraction. Calculate amount present by comparing peak areas from standards and samples.

Calculation

Calculate concentrations of AME, AOH, and TA in sample:

ng compound/g sample = $(AB)/C$

where A = concentration of compound, ng/mL; B = dilution of sample extract, mL; and C = amount of sample represented by extract (25 g).

Confirmation by Mass Spectrometry (MS)

Confirm presence of TA in sample by CI MS, using methane reagent gas at a source pressure of 1 torr. Repeatedly inject the extract containing >10 μg TA into liquid chromatograph and collect TA-containing effluent. To free TA from its zinc complex, dilute combined, collected TA solution with equal volume of 0.1N HCl and extract twice in separatory funnel with equal volumes of CH_2Cl_2. Let CH_2Cl_2 evaporate overnight. After redissolving TA in a small volume of CH_2Cl_2, introduce sample into mass spectrometer via direct insertion probe. Acquire full mass scan positive ion and negative ion spectra as probe is heated to 200°C. TA should give positive

ions 141 (M − 56), 198 (M + H), 226 (M + 29), and 238 (M + 41), and the negative ion 196 (M − H).

Alternatively, use gas chromatography/MS. Use on-column injection of 0.5 μL on 6 m methyl silicone capillary column. Operating conditions: temperatures (°C)—injector 230, column programmed 70–270 at 20°/min, transfer lines 240; 6 psi helium head pressure on column. Acquire full mass scan positive ion and negative ion spectra.

Results and Discussion

In developing this procedure, advantage was taken of the fact that TA, AOH, and AME strongly absorb at 280 nm while AOH and AME also exhibit strong fluorescence. TA forms a strong complex with Zn^{2+}, which eluted as a sharp peak from the liquid chromatograph (Figure 1), and when quantitated, showed a linear response between 1 and 1000 ng. For AOH and AME, the fluorescence detector gave a linear response at a concentration range of 0.05–5 ng.

Figure 2 shows the LC trace of a tomato sample spiked with TA at the 1.4 μg/g level. TA was confirmed by CI MS (Figure 3).

The positive ion and negative ion CI spectra of TA in Figure 3 were obtained by capillary gas chromatography/MS. This separated TA from the propyl homolog investigated by Joshi et al. (21). Confirmatory analysis for TA by using a direct insertion probe invariably produced a small response (<10% of the TA signal) for the propyl homolog (molecular weight 183) along with the TA response. Sample extracts were essentially free of other coextractives at the level of interest; therefore, the direct insertion probe was used for routine confirmation of TA.

The method was applied to catsup, tomato paste, and moldy tomato samples. For spiked catsup samples, the limit of determination was 25 ng/g for TA and 3 ng/g for AME. In catsup, an interfering peak prevented quantitation of AOH. The average recoveries for 8 catsup samples spiked at the 50 ng/g level were 74% (relative standard deviation (RSD) = 27%) for TA and 48% (RSD = 28%) for AME. At the 1000 ng/g level (4 samples), the average recoveries were 92% (RSD = 11%) for TA and 88% (RSD = 13%) for AME. Similar results were found with a smaller number of tomato paste samples fortified at the same levels.

A limited survey was made of moldy tomatoes collected from the processing lines of several catsup manufacturers. Of 142 samples analyzed, 69 were negative for TA, 28 had levels of 0.4–1.9 μg/g, and 45 had 2.0–70 μg/g. These samples were analyzed without use of the column cleanup step because this step is not essential when the method is directly applied to tomatoes. With tomatoes, the limit of determination for TA was estimated to be 0.4 μg/g, and the average recovery (4 determinations) at the 8 μg/g level was 55% (RSD = 8%). TA in 4 samples was confirmed by using the silica gel column and LC isolation steps and CI MS. Only a few of the 142 tomato samples showed any fluorescent peaks at the retention times of AOH and AME, indicating the possible presence of low levels of contamination by these metabolites; these were not confirmed.

In conclusion, the LC method described has good potential for detecting the use of *Alternaria*-contaminated tomatoes in the preparation of tomato products.

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Thin Layer Chromatographic Determination of Sterigmatocystin in Cheese

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A one-dimensional thin layer chromatographic method has been developed for determining sterigmatocystin in cheese. Cheese is extracted with acetonitrile–4% KCl (85 + 15). A simplified liquid-liquid partition cleanup is used, and the sample extract is passed through a cupric carbonate column for final purification. Sterigmatocystin is visualized by spraying the plate with aluminum chloride. The fluorescence of the spot is enhanced 10-fold by additional plate spraying with a silicone-ether mixture, enabling sterigmatocystin detection and quantitation at 2 and 5 μg/kg, respectively. Average recoveries were 88.3 and 86.4% at the 10 and 25 μg/kg levels, respectively.

Sterigmatocystin is a mycotoxin that has potent toxic and carcinogenic effects (1–3). This metabolite is produced by several species of *Aspergillus* and at least one species of *Bipolaris* (4). Sterigmatocystin occurs naturally in grains (5, 6), green coffee beans (7), fruit juices and fruit-based infant foods (8), and cheese (9, 10). Since sterigmatocystin-producing fungi are so ubiquitously distributed, this metabolite must be considered as an important contaminant in these commodities as well as in other foods and feeds. Thus, interest was aroused in analyzing cheese for sterigmatocystin to determine levels and incidences of contamination.

Methods have been published for the determination of sterigmatocystin in grains and cereals (11–13) and have been used for other commodities; however, these methods were found to be unsatisfactory for this project. The method of van Egmond et al. (14), which is essentially their earlier method (15) with modifications, had been developed for the determination of sterigmatocystin in cheese, reportedly with a 5 μg/kg limit of detection. We tested this method and concluded that it was complex and time consuming, and had a relatively high limit of detection along with poor spiked sample recoveries. Experimental data collected during the method evaluation indicated the potential for the development of a more satisfactory procedure. The thin layer chromatographic (TLC) method described here resulted.

METHOD

Apparatus

(a) *Chromatographic tube.*—Plain, 22 × 250 mm.

(b) *TLC plates.*—Precoated, Sil G-25 HR No. 66–14–600–6 (Brinkmann Instruments, Inc., Cantiague Rd, Westbury, NY 11590). Do not activate plates.

(c) *TLC apparatus.*—Developing tank with cover for use with 20 × 20 cm glass plates; spotting template; 10 μL syringe; longwave 15 watt UV lamp (use with absorbing eyeglasses) or Chromato-Vue cabinet (Ultra-Violet Products, Inc., San Gabriel, CA 91778) equipped with one or two 15 watt lamps; if available, a thin layer plate densitometer with fluorescent capability, excitation wavelength 365 nm and emission wavelength >400 nm (Shimadzu High-Speed TLC Scanner Model CS-920, Shimadzu Scientific Instruments, Inc., 7102 Riverwood Dr, Columbia, MD 21046).

(d) *TLC plate sprayer.*—Kontes, K-422540.

(e) *Blender.*—Waring, equipped with 1 L jar and lid.

(f) *Filter paper.*—Whatman 2V folded circles, 24 cm.

(g) *Vials.*—5 mL conical, tapered inside, with Teflon-lined screw cap (No. 13053, Applied Science Laboratories, Inc., State College, PA 16801).

Reagents

(a) *Solvents.*—Reagent grade acetonitrile, hexane, methylene chloride, benzene, methanol, acetic acid, and anhydrous ethyl ether. (*Caution:* Benzene is a suspected carcinogen. Use necessary safety measures when handling this chemical.)

(b) *Silicone DC-200.*—12 500 centistokes (No. 08107, Applied Science Laboratories Inc.).

(c) *Silicone-ether spray.*—Silicone–anhydrous ether (18 + 82). If weighing silicone is more convenient, 1 g = 1 mL.

(d) *Potassium chloride solution.*—Dissolve 4 g KCl in 100 mL water.

(e) *Aluminum chloride solution.*—Dissolve 15 g $AlCl_3$ ($AlCl_3 \cdot 6H_2O$) in ethanol and dilute to 100 mL.

(f) *Calcium chloride.*—Anhydrous, granular (8 mesh) (No. 1309, J. T. Baker). Prepare in advance $CaCl_2$–water (1 + 2, w/v). Hasten solution by placing in ultrasonic bath. Remove slight cloudiness by filtering through Whatman 2V paper.

(g) *Cupric carbonate, Cu (II).*—Reagent grade powder (J. T. Baker).

(h) *Diatomaceous earth.*—Hyflo Super-Cel.

(i) *Sterigmatocystin standards.—Stock standard.*—Prepare according to sec. **26.133(e)** (16). *Working standard.*—Dilute stock standard to ca 1 ng/μL benzene.

(j) *Boiling chips.*—SiC. Float off fines and extraneous particles with water, wash with acetone, and dry.

Extraction

Weigh 36 g composited sample into 1 L Waring blender jar. Add 170 mL acetonitrile and 30 mL KCl solution; secure lid and blend 3 min at high speed. Decant extract through Whatman 2V paper. Transfer 50 mL filtrate to 250 mL separatory funnel, add 50 mL $CaCl_2$ solution, vigorously shake ca 30 s, and let stand until layers separate; separation usually occurs within 5 min.

Discard bottom layer. Remove excess water from inside the stem of the separatory funnel by drying with laboratory wiping tissue, such as Kimwipes. Place a small wad of glass wool into the stem of the separatory funnel to serve as a filter and drain the remaining phase into a second separatory funnel. Add 50 mL water to remaining phase and mix. Add 50 mL hexane, vigorously shake ca 1 min, and let layers separate. Transfer bottom layer to another 250 mL separatory funnel. Extract, using 50 and 25 mL portions of methylene chloride, by shaking first extraction 1 min and second extraction 10 s. Again, complete separation usually occurs within 5 min. Collect methylene chloride extracts separately and save for column chromatography.

Column Chromatography

Loosely place glass wool plug in bottom of chromatographic tube and add ca 15 g anhydrous sodium sulfate. Add 4 g cupric carbonate–diatomaceous earth (1 + 2); lightly pack column by holding in upright position and by allowing it to drop gently on its tip onto book or writing pad. Add 20 g sodium sulfate to top of column.

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Sequentially pass methylene chloride extracts through column, beginning with 50 mL portion. Collect that eluate and each successive eluate in 250 mL Phillips beaker. Add 25 mL portion to column when first portion has drained to below top layer of sodium sulfate. Rinse column with 10 mL methylene chloride.

Add 4–5 SiC boiling chips to Phillips beaker and evaporate solvent nearly to dryness on steam bath under gentle stream of nitrogen. Cool to room temperature and quantitatively transfer with small portions of methylene chloride to 5 mL vial. Evaporate solvent to dryness under gentle stream of nitrogen. Save sample residue for TLC.

Thin Layer Chromatography

Add 100 μL benzene (screen ≤10 ppb) or 200 μL benzene (screen ≤25 ppb), cap vial, and vigorously mix ca 30 s, preferably with Vortex or similar shaker. Puncture Teflon liner to accommodate needle of 10 μL syringe. Spot two 5 μL portions and one 10 μL portion of sample extract. Overlay one 5 μL sample spot with 5 μL sterigmatocystin working standard. Spot 2, 4, 6, 8, and 10 μL working standard. Let spots air dry. Develop plate with benzene–methanol–acetic acid (85 + 10 + 5) for 45 min. Solvent front travels 13–15 cm in 45 min. Remove plate from developing tank and air dry 10–15 min (5 min at 40°C in forced draft oven). Using $AlCl_3$ reagent, spray plate evenly across upper half of developed area. Do not spray to point of visible wetness. Heat plate at 110°C ca 5 min. Let plate cool slightly, but while still warm spray same area with silicone-ether mixture, making several passes over area, but do not spray to point of visible wetness. Place plate in 110°C oven ca 3 min. Repeat silicone-ether spraying and drying before quantitation of sterigmatocystin. Examine plate under longwave UV light at 365 nm. Spots of sterigmatocystin will give bright yellow fluorescence. If sample pattern exhibits yellow fluorescent spots at same R_f as standard, compare fluorescent intensity of sterigmatocystin spots of sample with those of standard spots.

If resolution of sterigmatocystin is inadequate for visual or instrumental quantitation, use alternative TLC system: Deactivate silica gel of TLC plate by placing plate along with several small beakers (100–250 mL) containing water in sealed container, such as developing tank, and let stand ≥1 h before using.

Table 1. Recovery of sterigmatocystin added to Gouda cheese

Detn No.	Found, μg/kg	Rec., %
	10 μg/kg Level	
1	0, control	
2	10.0	100
3	9.9	99
4	8.4	84
5	8.6	86
6	7.9	79
7	8.2	82
Mean	8.8	88.3
Std dev.	0.89	
Coeff. of var., %	10.2	
	25 μg/kg Level	
1	0, control	
2	27.6	110
3	21.4	86
4	19.7	79
5	20.2	81
6	20.0	80
7	20.8	83
Mean	21.6	86.4
Std dev.	2.99	
Coeff. of var., %	13.9	

Spot plate as previously described and develop using benzene–methanol (95 + 5). Solvent front travels 13–15 cm in 45 min.

Calculation

Calculate concentration of sterigmatocystin:

$$\mu g/kg = ng/g = (S \times Y \times V)/(X \times W)$$

where S = μL standard equal to unknown, Y = concentration of standard (ng/μL), V = final volume of sample extract (μL), X = μL sample extract spotted giving fluorescent intensity equal to S, and W = g sample in final extract (9 g).

Results and Discussion

Table 1 shows the percentage recovery of sterigmatocystin from spiked Gouda cheese after extraction and cleanup, as measured by one-dimensional TLC. The mean recoveries of sterigmatocystin added at levels of 10 and 25 μg/kg were 88.3 and 86.4%, respectively. The coefficients of variation were 10.2 and 13.9%.

While trying to resolve the problem of sterigmatocystin spots fading on TLC, we noticed that spots, particularly those of 30 ng and less, were very visible, especially when the plate was still warm; however, the spots faded rapidly upon cooling of the plate. We also observed that spot intensity could be regenerated by reheating the plate. This fading was unlike fading shown by aflatoxins, in that aflatoxin fading is minimized when plates are protected from light and, in some cases, from atmospheric contaminants such as ozone. With sterigmatocystin, we surmised that the activity of the silica gel was causing the observed behavior. We concluded that the problem would be solved if the silica was held in an activated state, which could be accomplished by sealing the plate with paraffin or silicone. Various mixtures of silicone–anhydrous ether were applied by spraying before arriving at the mixture described here.

In addition to sealing the plate, the silicone also enhanced the fluorescence of the sterigmatocystin spots approximately 10-fold. This type of fluorescent spot enhancement had been noted earlier by other researchers (17), who used viscous organic solvents such as mixtures of liquid paraffin and *n*-hexane as a spray.

No significant loss in fluorescent intensity of sterigmatocystin was observed in plates that were stored unprotected from light and moisture for 2 weeks.

A sterigmatocystin standard curve gave a linear response in the range from 3 to 106 ng, with a correlation coefficient (*r*) of 0.9994. One ng sterigmatocystin standard is easily detected, while 3 ng represents the lowest amount quantitated at a signal-to-noise ratio of 3:1.

The proposed method is simple, practical, rapid, economical, and precise. Incorporation of calcium chloride solution into the sample extract before hexane defatting and methylene chloride partitioning solved emulsion problems previously encountered when using other methods. The elimination of emulsion formation during separatory funnel cleanup and partitioning has possibly provided the breakthrough necessary to improve analytical methods used for the determination of other mycotoxins. The concentration of calcium chloride used may vary among commodities. The minimum concentration required to be effective in this method has not been thoroughly investigated. Remaining TLC interferences are eliminated by passing the methylene chloride extract through the cupric carbonate column. Further work is in progress on the use of the method to determine other mycotoxins.

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Assessment of Extraction Procedures in the Analysis of Naturally Contaminated Grain Products for Deoxynivalenol (Vomitoxin)

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A comparison of 2 extraction solvent systems (acetonitrile–water, 21 + 4 and methanol–water, 1 + 1) and 3 mixing apparatus (high-speed blender, wrist-action shaker, and mechanical stirrer) was carried out for different extraction time periods. Methods were evaluated using uncontaminated corn spiked with pure deoxynivalenol (DON), field-inoculated (*Fusarium graminearum*) corn, and uncontaminated and naturally infected wheat in swine diets. After sample extraction, aliquots were passed through alumina-charcoal cleanup columns, evaporated to dryness, dissolved in 8% aqueous methanol, and injected onto the liquid chromatograph. Results confirm published reports of recoveries from DON-spiked samples; however, longer extraction times (≤ 120 min) were required for naturally contaminated samples. Use of the high-speed blender resulted in faster extractions, but in our laboratory more samples could be more conveniently extracted simultaneously with the wrist-action shaker or mechanical stirrer. Less carryover (co-extraction) of interfering contaminants was observed when acetonitrile–water was used vs methanol–water. Results emphasize the importance of careful evaluation of extraction procedures with not only spiked samples but also naturally contaminated samples to establish extraction times required for maximum deoxynivalenol recoveries.

Deoxynivalenol (DON, vomitoxin) is a toxic fungal metabolite belonging to a class of mycotoxins called trichothecenes (1, 2). DON is produced by *Fusarium* mold which infects crops such as wheat, corn, and barley. When contaminated grain was mixed and fed to livestock, it was reported that decreased feed consumption, decreased weight gain, feed refusal, vomiting, and diarrhea occurred (3). Although swine appear to be sensitive to ingestion of DON, they can tolerate dietary levels up to 2 mg DON/kg feed before serious health effects occur (4–6). Research to date indicates that poultry and dairy cattle can consume higher DON concentrations without adverse effects on health and performance (7–10). In 1980, DON was identified in wheat produced in eastern Canada (11). In 1982, unusual weather conditions in Midwestern United States led to scabby wheat and mycotoxin contamination (12). Extensive surveys were initiated quickly to define the nature and extent of contamination and evaluate the safety of the use of the infected wheat.

Analysis of contaminants in grains and plant products is not simple. Many variables must be carefully controlled, such as proper sampling from large quantities of starting material, and handling, storage, preparation, and subsampling of initial samples to ensure that analysis is carried out on a truly representative sample of the test material (13, 14). Analysis is further complicated by possible heterogeneity of the starting material, and the presence of contaminants in the storage or delivery systems (15). On the other hand, during the analysis, variables such as precision, accuracy, and detection limit (16) need to be assessed to assure that quantities measured are representative of the starting material and that the analytical errors are minimized (17, 18). Over the past several years, we have found that the final amount of sample used for chemical analysis is very important. Precision improved markedly when sufficient quantities (50–200 g) of samples were used (unpublished results).

Initially, the gas chromatographic-electron capture (GC-EC) detector method developed by Scott et al. (19) was widely used for quantitative analyses; although more recently, other laboratories have reported GC methods (20–23) as well as thin layer chromatographic (TLC) (24, 25), gas chromatographic-mass spectrometric (GC-MS) (19, 26, 27), MS-MS (28), and liquid chromatographic (LC) (29–32) methods. There is definite interest, however, both in our laboratory (27) and elsewhere (R. M. Eppley, Food and Drug Administration, Washington, DC (1984) personal communication) concerning the considerable variation in results seemingly demonstrated between laboratories or procedures or both. The precision and accuracy of the methods used are probably not limited by the analytical instrumentation available, but by the pre-chromatographic workup of samples, including sampling technique (13), and by the diverse extraction and cleanup procedures used by various laboratories involved in mycotoxin analyses (14–16).

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When developing trace analytical methods involving nonspecific detectors, e.g., LC spectrophotometric detectors, it is imperative to use selective cleanup procedures. Regardless of the method used, a major problem is the co-extraction of endogenous compounds which subsequently interfere in LC analyses and hamper attempts to quantitate compounds in the submicrogram range. We considered it necessary to compare extraction methods and to define optimum parameters for quantitative isolation of DON from grain products before LC analyses.

This report presents results from a study of alternative extraction procedures for DON that may partially explain some of the variations among quantitative results reported using different analytical methods and among different laboratories (27).

Experimental

Apparatus and Reagents

(a) *Glassware.*—Before use, glassware was thoroughly washed and heated 2 h in a muffle furnace at 500°C as described previously (33).

(b) *Liquid chromatograph.*—Spectra-Physics Model SP-8000-03 microprocessor-based, fitted with temperature-programmed oven (30°C); autoinjector with 100 μL sample loop; UV detector with auto-zeroing device, cathode ray tube display unit; and autosampler as described previously (33).

(c) *Ultraviolet-visible detector.*—Variable wavelength, Spectro-Flow 773 absorbance detector (Kratos Analytical Instruments, Westwood, NJ) set at 220 nm.

(d) *LC column.*—Stainless steel, 25 cm × 4.6 mm id, packed with reverse phase RP-18, 5 μm OD-5A Spheri-5 (Brownlee Labs, Santa Clara, CA). Guard column (Part No. 84550, Waters Scientific Ltd, Mississauga, Ontario), filled with Spherisorb S5 ODS, 5 μm OD-5 (Cat. No. 1610 0310, Phase Separations, Hauppage, NY), was inserted between injector and LC column.

(e) *LC mobile Phase.*—Methanol–water (2 + 23). Methanol (LC grade) was filtered through FH-0.5 mM filter (Millipore Ltd., Mississauga, Ontario); water was double distilled in glass and filtered through Millipore filter HA-0.45 mM.

(f) *DON standard solution.*—20.15 μg DON/mL acetone; pure deoxynivalenol standard was obtained from P. M. Scott, Health and Welfare Canada, Ottawa, Ontario, Canada.

(g) *Grinder.*—Thomas-Wiley laboratory mill, Model 4 (Arthur H. Thomas Co., Philadelphia, PA).

Sample Preparation

Analyses were carried out on 2 swine diets containing naturally contaminated wheat (2.5 mg DON/kg diet) and a sample of heavily contaminated, field-inoculated (*Fusarium graminearum*) corn (170 mg DON/kg) from ARC (Animal Research Centre) feeding trials. Noncontaminated samples of both grain types were used for controls and spiked samples.

Aliquots (20 kg) of each coarsely ground starting material (swine diets and corn) were well mixed and stored at 4°C. Before analysis, samples of 5 kg wheat and 10 kg corn were ground in a Wiley mill and passed through No. 20 mesh screen. Analyses were conducted on representative 50 g aliquots.

Sample Spiking

As part of the recovery studies, noncontaminated corn and swine diets were spiked with pure DON. An acetone solution of DON was added dropwise and was thoroughly distributed within the sample. The acetone was removed by passing a stream of air over the sample for 1 h at room temperature.

Extraction Methods

Two solvent systems, acetonitrile–water (21 + 4) and methanol–water (1 + 1) were evaluated for their efficiency to extract DON from mold contaminated and spiked samples of finely ground corn and wheat-based swine diets.

Three types of commonly used mixing apparatus were evaluated for their effectiveness during the extraction procedure: high-speed blender (Waring 700-B Blendor), wrist-action shaker (Wrist-Action Shaker, Burrell Corp., Pittsburgh, PA), and a mechanical stirrer equipped with a stainless steel propeller blade. Samples were extracted with either of the above-mentioned solvent systems: high-speed blender, 400 mL acetonitrile–water or 250 mL methanol–water; wrist shaker, 200 mL acetonitrile–water in 500 mL ground glass-stopper Erlenmeyer flask; and mechanical stirrer, 400 mL acetonitrile–water in 32 oz multipurpose, white polystyrene, disposable container with tight-fitting lid (Miles Laboratories, Inc., Lab-Tek Div., Naperville, IL).

Extraction Sampling

Aliquots of the extracts were taken at different time intervals by interrupting mixing briefly. Solids were allowed to settle 1 min, and 1 mL extract was transferred quantitatively to an alumina-charcoal cleanup column (Figure 1). In the mechanical stirring experiments, the container with lid, including sample, solvent, and propeller were weighed before mixing. Solvent was added to the container to bring contents up to the initial weight before extract aliquots were taken, to eliminate errors caused by solvent evaporation.

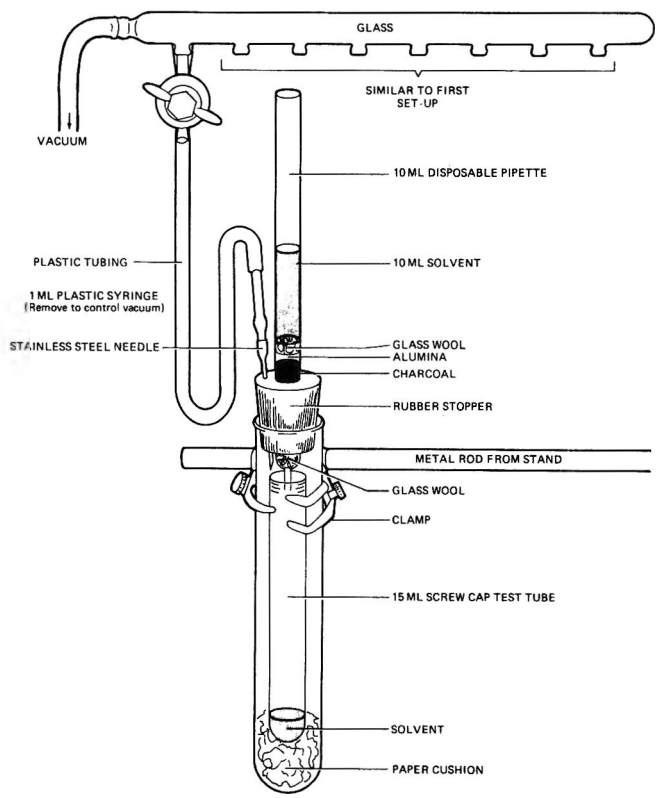

Figure 1. Vacuum system for cleanup of extracts on alumina-charcoal columns, showing collection of eluate under vacuum. Multiple samples can be run simultaneously by addition of more column units to vacuum ports at top of apparatus.

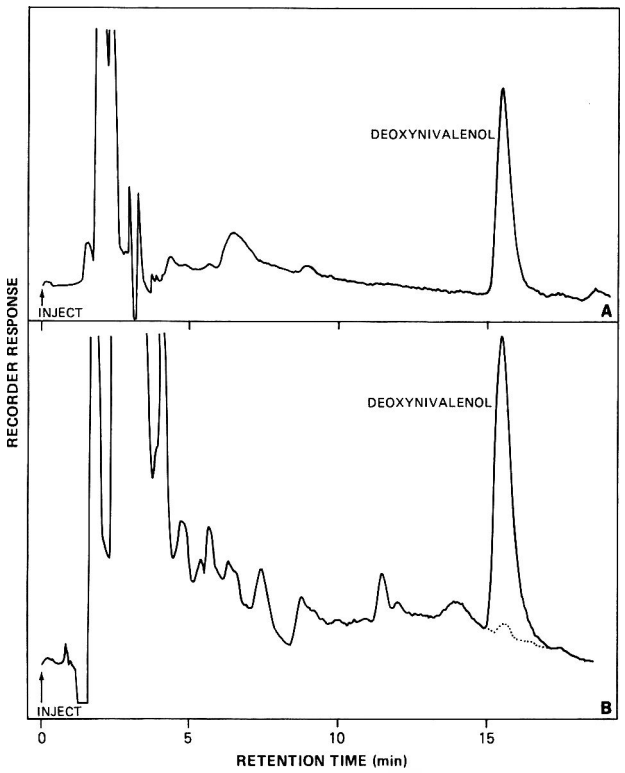

Figure 2. LC chromatograms of DON standard (25 ng injected) (A) and naturally contaminated wheat (B) containing 1.75 mg DON/kg. Dotted line under DON peak indicates wheat blank.

Alumina-Charcoal Column Cleanup

Nonactivated charcoal (0.75 g of Darco G-60, 7-E343, Baker Chemical Co., Phillipsburg, NJ) was added to a 10 mL disposable glass pipet (8 mm id, 13-678-27F, Fisher Scientific, Pittsburgh, PA) with a silanized glass-wool plug in the tip (Figure 1). To facilitate column loading, the upper restriction of the pipet was removed. While vacuum was applied to the tip of the column in a vertical position and with gentle tapping, the charcoal was compacted to ca ⅔ of the original volume in the column. Similarly, neutral alumina (0.7 g of 70–230 mesh, Merck M 01077-36, BDH Chemicals, Toronto) was added above the charcoal. A glass-wool plug was fitted above the alumina layer, and the column was prewashed with 15 mL acetonitrile–water (21 + 4). All subsequent steps were also carried out under slight vacuum, and at no time was the column permitted to run dry.

To determine when DON eluted from the column, 4.0 mL DON standard (0.025–25 μg/mL acetonitrile–water, 21 + 4) was applied quantitatively to the prewashed column, and 4.0 mL eluate was collected. The flow rate through the column was maintained at 1 drop ca every 10 s by adjusting vacuum pressure. More solvent was added to the top of the column, and additional 5 mL fractions were collected and analyzed for DON by LC. For the current lot of charcoal, it was determined that a total of 14 mL eluate was required for quantitative elution of DON through this cleanup column.

LC Quantitation

The eluate (14 mL), collected in a round-bottom test tube, was evaporated to dryness (N_2, 50°C). The resulting residue was dissolved in an appropriate volume of 8% aqueous methanol to obtain a 10–15 cm peak. The sample aliquot was added to an LC sample tube, capped, and placed in the LC autosampler for analyses (33). DON standard solutions were prepared and analyzed with unknown samples (Figure 2) to quantitate by comparing ratios of the peak heights of unknowns vs peak heights of standards. All plots were linear over a 0.5–100 ng DON range of amount injected.

Results and Discussion

Our underlying objective was to develop a simple extraction procedure for DON analysis that would be quantitative and allow multiple samples to be processed automatically. In earlier work with mycotoxins, we reported GC and LC methods for zearalenone and its derivatives in grain products (34, 35). When we initiated research on the safety of DON in animal feeds, it soon became obvious that a highly sensitive method was required to analyze DON levels in suspected feed samples submitted by producers experiencing problems with livestock and poultry and in diets prepared for feeding trials (10, 11).

We chose to develop an LC method because new ultraviolet-visible detectors are very sensitive and the technique does not require derivatization as do GC procedures and can be readily adapted to multisample, automated analysis. An LC method, however, requires efficient, yet selective, extraction of DON to be suitable for routine work.

Several methods are available for extraction of DON from wheat and corn samples, but they use 2 solvent systems extensively. In 1981, Scott et al. (19) used methanol–water (1 + 1) and 5 min high-speed blending. Bennet et al (20) used the same solvent system, but extracted 30 min on a wrist-action shaker. Ehrlich et al. (29) developed a method with methanol–water (17 + 3), although no length of extraction time was mentioned. In 1984, Chang et al. (31) used acetonitrile–water (21 + 4) and 3 min high-speed blending. In a recent collaborative study (R. M. Eppley, Food and Drug Administration, Washington, DC (1984) personal communication), Eppley evaluated a method using acetonitrile–water (21 + 4) and 30 min vigorous shaking. Romer et al. (25) extracted samples with acetonitrile–water (21 + 4) and mixed them in a blender for 5 min or on a gyrotory shaker for 60 min.

Initial work in our laboratory demonstrated that acetonitrile–water (21 + 4) solvent was better than methanol–water for LC analysis because it resulted in less co-extraction of interfering contaminants. Therefore, simpler, faster cleanup procedures could be used in the analysis. The method eval-

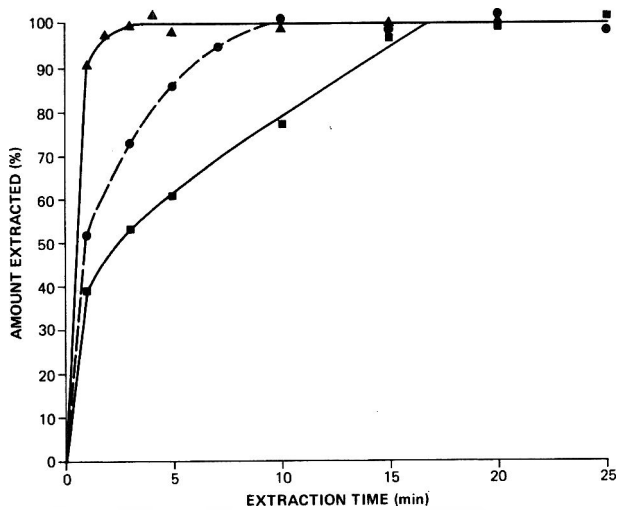

Figure 3. Extraction of DON from spiked (▲) and naturally contaminated swine diet (■) with acetonitrile–water by Waring blender as a function of time. For reference, the naturally contaminated swine diet was also extracted (●) using Scott's methanol–water procedure (19).

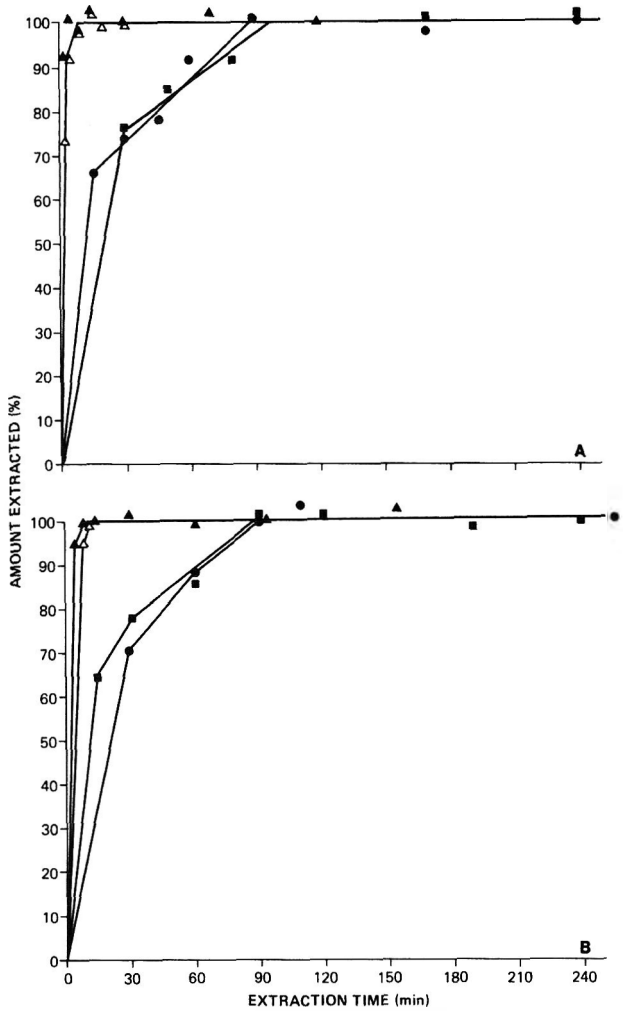

Figure 4. Extraction of DON with acetonitrile–water from spiked (▲, 2.5 mg DON/kg uncontaminated swine diet; △, 92 mg DON/kg corn) and naturally contaminated grain products (■, 2.5 mg DON/kg naturally contaminated swine diet; ●, 170 mg/kg corn), using wrist shaker (A) and mechanical propeller stirrer (B).

uated was a modification of earlier methods reported by Chang et al. (31) and Romer et al. (25). Preliminary studies indicated that incomplete extractions might cause some of the variability observed in our results and in those of other laboratories (27). To clarify the matter, the effect of extraction duration on calculated DON concentrations in spiked and naturally contaminated grain products was evaluated using a high-speed blender, wrist shaker, and mechanical propeller stirrer. Aliquots were taken at different time intervals of extraction, and DON was measured. The methanol–water (1 + 1) extraction procedure of Scott et al. (19) was used as a reference method.

A comparison of the rate of DON extraction from a spiked, swine diet sample vs a comparable diet containing naturally contaminated wheat using a high-speed blender and acetonitrile–water solvent is shown in Figure 3. The results confirm data published by Chang et al. (32) who reported that after 3 min blending, all DON was extracted from the spiked sample. In our laboratory, however, only 53% of DON in the naturally contaminated sample was extracted within the same 3 min. A minimum of 16 min was required for complete DON extraction from the naturally contaminated sample. As a comparison, 5 min blending by the Scott's procedure (20) extracted 86% of DON from the naturally contaminated sample.

When a wrist shaker was used with the acetonitrile–water solvent, DON in the spiked samples (both high and low levels) was extracted in 7–10 min, whereas DON in the naturally contaminated sample required a minimum of 90 to 120 min to ensure complete extraction (Figure 4A). In comparison, when vigorous mechanical stirring was used with the acetonitrile–water solvent (Figure 4B), DON in the spiked sample was again extracted within 10 min, while the extraction of DON from the naturally contaminated material required 90 min.

The results emphasize the importance of careful laboratory evaluation of extraction procedures before routine use. Differences in sample composition, grinding technique, and mechanical mixers can alter the minimum time period needed for complete extraction. Use of spiked samples is important to test the efficiency of a given method; however, spiked samples may not always be adequate substitutes for naturally contaminated samples (36). Spiking probably does not represent the actual distribution of DON within naturally contaminated grains. The results in this report indicate that DON in spiked samples is readily extracted because the mycotoxin is located at or near the surfaces of granules. In contrast, milling studies with naturally contaminated wheat (37) suggest that DON is well distributed throughout wheat kernels. This would explain, at least partially, why longer extraction periods are needed to remove DON from deeper within the sample particles.

In 1983, Rothberg et al. (27) compared results for the quantitation of DON by various analytical methods (GC-EC; combined TLC, GC and MS; and negative chemical ionization-mass spectrometry (NCI-MS)) for samples of contaminated corn, wheat, and mixed feed. Considerable variation was observed between laboratories regardless of method used. Data for DON extraction as a function of time (Figures 3 and 4) offer an explanation for some of the intra- and interlaboratory variation. If the extraction times, and subsequent filtration or separation steps, were not carefully controlled, variations in DON results would occur when the extraction periods were less than that required for complete extraction. Variation might also occur as a result of different extraction rates for different samples. The good agreement between the NCI-MS and Scott's GC-EC method can be partially attributed to use of the same extraction solvent (methanol–water) for both methods (27).

The data presented in this report for the effect of extraction time on the recovery of DON have much broader implications. Researchers planning or developing analytical methods for biological samples should carry out initial studies using samples spiked with pure standards in noncontaminated material. Every effort should be taken to ensure effective dispersion of the standard throughout the sample to be analyzed. The results will provide background information on recoveries, and on interferences resulting from superimposed or poorly resolved peaks. Once the method appears to be working well with spiked samples, analyses should be repeated with naturally contaminated biological samples at different extraction time periods to ensure complete extraction.

The following comments concern sample cleanup before LC analysis: We have had success with extracts equivalent to ≤0.8 g grain sample passed through nonactivated charcoal-alumina columns that are prepared under vacuum; DON recoveries were consistently in the 90–100% range. When larger quantities of extracts were used (10–20 mL extracts from 2–4 g sample), the cleanup column was overloaded which resulted in increased interference by contaminating peaks and decreased recoveries of DON.

The extraction apparatus indicated in Figure 1 provides an effective and simple cleanup method. Because no solvent

transfer of the dried extracts to other containers is necessary, extraction time is minimized and precision is improved.

In summary, after examination of several LC methods currently in use, we believe the following procedure is well suited to rapid, sensitive, and quantitative analysis of DON in naturally contaminated grain products: samples finely ground, 50 g subsample taken for analysis; extraction with 250 mL acetonitrile–water (21 + 4) on wrist shaker for 120 min (high speed blending and mechanical stirring are adequate if enough time is allowed); cleanup of 4 mL extract on charcoal-alumina column; and LC analysis.

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PRESERVATIVES

Liquid Chromatographic Analysis of Dehydroacetic Acid and Its Application to Wines

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A simple liquid chromatographic procedure is described for determination of the preservative dehydroacetic acid (DHA) in wine. No cleanup procedure is necessary; the sample is injected onto an NH_2 column without further pretreatment. The mobile phase is a 9 + 1 mixture of acetonitrile–sodium acetate buffer adjusted to pH 4 with acetic acid. Total run time is less than 10 min. Dehydroacetic acid is determined by using the absorbance at 307 nm; the detection limit is estimated to be 0.2 ppm. The method may also be suitable for detecting DHA in a variety of foodstuffs in low concentrations.

In 1947, Coleman and Wolf (1) discovered the antimicrobial action of dehydroacetic acid (DHA) (3-acetyl-6-methyl-2*H*-pyran-2,4(3*H*)-dione). This compound appears to be effective even in the high pH range, but has never acquired great significance because of its relatively high toxicity. Methods were described in a number of publications for the determination of DHA in wine (2–5), beer (6), cheese (2, 7), and squash (8, 9).

According to the *U.S. Code of Federal Regulations*, Title 21, §172.130, DHA and its sodium salt are only permitted for treating cut or peeled squash. The maximum permissible residue is 65 ppm. In Europe, dehydroacetic acid is not permitted as a food preservative. However, Haller and Junge (3) reported the possible use of DHA as a preservative in wines.

Various techniques have been used to determine DHA in food, including gas (10) and liquid chromatography (11). All the techniques described require a number of manipulative steps in the sample cleanup. In this paper we describe a sensitive liquid chromatographic method for determination of DHA in wine, without any necessary sample cleanup.

Experimental

Apparatus and Reagents

(a) *Liquid chromatograph.*—Perkin-Elmer, consisting of Series 3B pump, Model LC-75 variable wavelength detector (Perkin-Elmer, Norwalk, CT), recorder BD40 (Kipp en zonen, Delft, Holland), and Rheodyne Model 7125 injector equipped with 20 μL loop (Rheodyne Inc., Berkeley, CA). Chromatographic conditions: flow rate, 2.0 mL/min at 20 bar; detector wavelength 307 nm, detector sensitivity 0.08 AUFS for 5 ppm DHA; column temperature ambient.

(b) *Column.*—LiChrosorb 10 NH_2, 25 cm × 4.6 mm id (Merck, Darmstadt, FRG).

(c) *Buffer solution.*—Prepare 15mM sodium acetate solution in water. Adjust to pH 4.0 with acetic acid, reagent grade.

(d) *Mobile phase.*—Acetonitrile–sodium acetate buffer (9 + 1 v/v). Pass eluant through Millipore sandwich with 0.45 μm membrane and degas in ultrasonic bath. Flow rate, 2 mL/min.

Procedure

Let chromatographic system equilibrate ca 1 h. Inject, in duplicate, 20 μL 5 ppm standard in water into liquid chromatograph, at sensitivity of 0.08 AUFS. Start with buffer concentration of 15mM in mobile phase. At same sensitivity, inject wines to be investigated, without further pretreatment.

If it is necessary to increase the k' of DHA, decrease the buffer concentration.

Results and Discussion

The chromatographic separation technique presented here is based on a combined reverse phase liquid partition and ion exchange. Most of the matrix components of the wine elute according to a reverse phase mechanism, but DHA is separated merely by ion exchange. This makes it possible to elute most of the matrix components before DHA. Variation of the acetate concentration allows one to position the DHA peak in the chromatogram without influencing the position of other peaks.

Figure 1 illustrates influence of the acetate concentration on the k' of DHA. In this application, the buffer concentration was varied from 5.0 to 15.0mM sodium acetate at pH 4, depending on the nature of the sample matrix. Response was shown to be linear over 3 orders of magnitude (0.5–500 ppm).

Figure 2 shows examples of white and red wines, port, and sherry which have been spiked with 5 ppm DHA. All samples were injected directly onto the column.

The method described does not use an internal standard and therefore quantitation depends solely on injection volume reproducibility. The standard deviation for repetitive injections of a standard solution was always <2%. The reproducibility of response for a red wine sample spiked with 5 and 50 ppm, respectively, was measured by injecting the sample 10 times. The mobile phase consisted of a 9 + 1 mixture of acetonitrile–7.5mM sodium acetate, pH = 4. The coefficient of variation ($100s/\bar{x}$ %, where s = standard deviation and $\bar{x}$

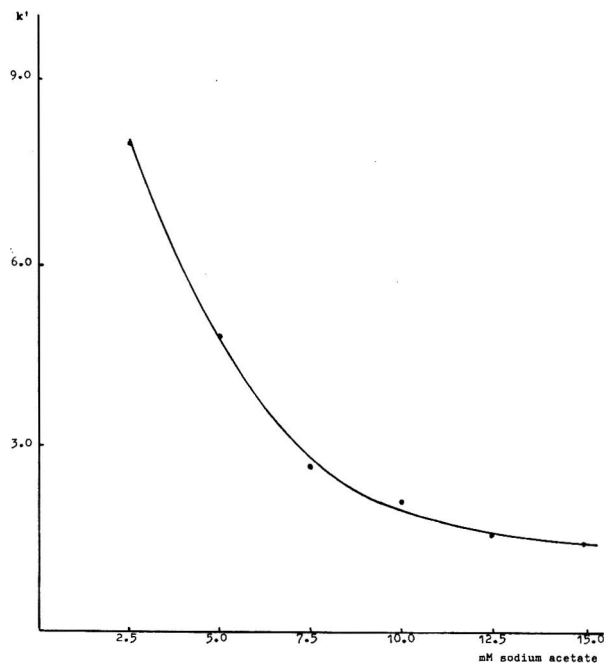

Figure 1. Influence of acetate concentration on capacity factor k' of DHA.

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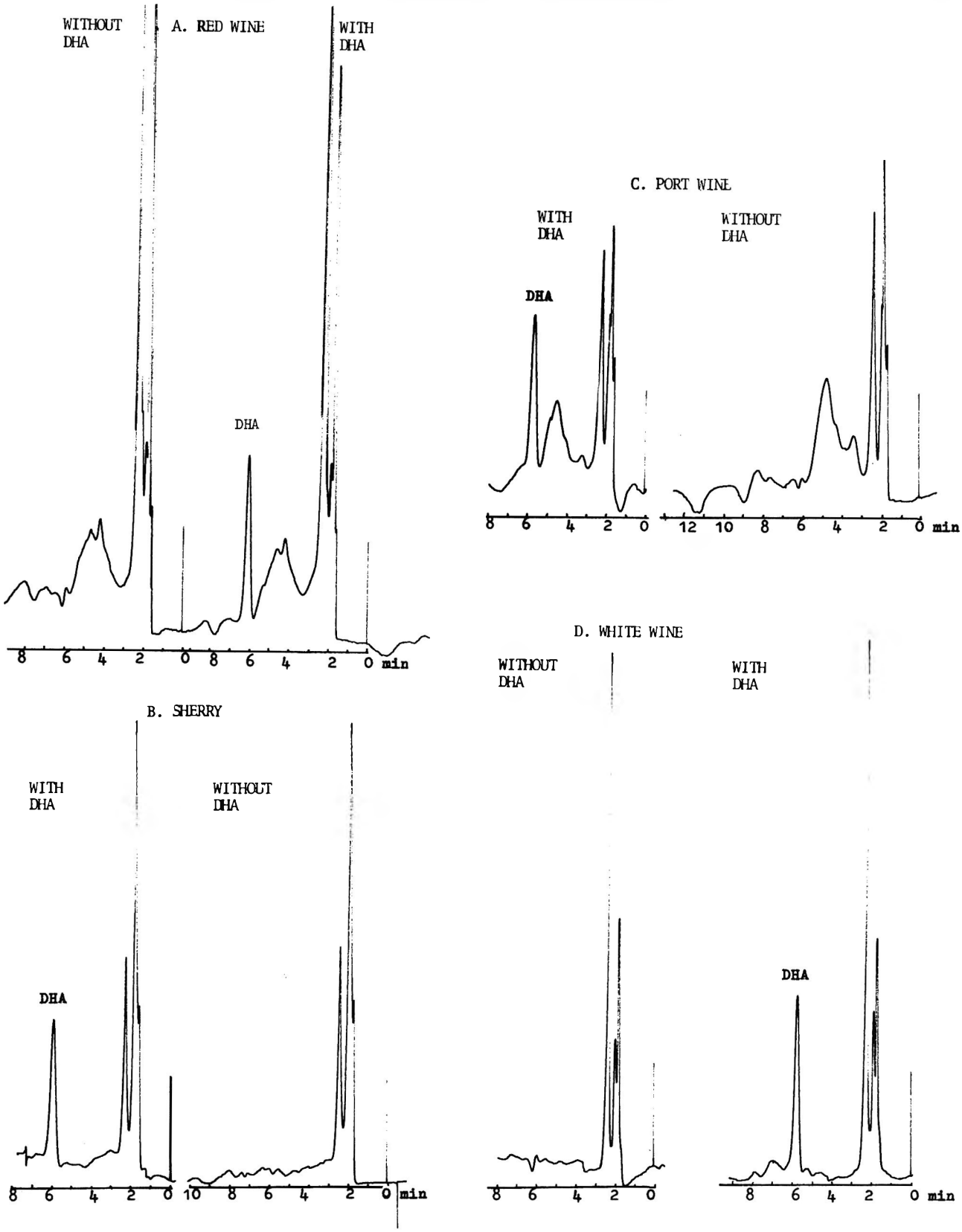

Figure 2. Chromatograms of wine samples spiked with 5 ppm DHA. Acetate concentration, 7.5mM.

= mean) was 4.5% for the 5 ppm and 3.5% for the 50 ppm spiked wine. The 95% confidence interval of the mean was 5.0 ± 0.2 ppm and 50.0 ± 1.2 ppm respectively, measured as $\bar{x}$ (95%) = $t \cdot s/\sqrt{n}$ where t is the Student t-value. These results could be improved by using a lower buffer concentration in the eluant.

Samples of 4 different types of wines were spiked at 3 levels (Table 1). The average recovery was 101.3%. The detection limit was estimated to be 0.2 ppm ($S = 3N$), using a 20 μL injection loop.

During this survey, 6 white, 7 red, 1 rosé, and 1 sparkling Californian wines were analyzed for DHA. None of these

Table 1. Recovery data for 4 samples of wine (mobile phase, 9 + 1 mixture of acetonitrile–7.5mM sodium acetate, pH = 4)

Wine	DHA added, ppm	DHA found, ppm	Rec., %
	100	99.0	99.0
Red wine	50	49.1	98.1
	5	5.1	102.6
	100	92.9	92.9
White wine	50	49.1	98.1
	5	5.1	101.8
	100	100.9	100.9
Sherry	50	52.4	104.8
	5	5.1	102.6
	100	100.8	100.8
Port wine	50	54.0	108.6
	5	5.3	105.1
Av.			101.3

wines showed the presence of this preservative. The proposed method should also be tested to determine DHA in other foodstuffs in low concentrations. The method is simple and rapid, especially for wine where no pretreatment is necessary.

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DRUGS IN FEEDS

Liquid Chromatographic Determination of Carbadox Residues in Animal Feed

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A liquid chromatographic (LC) method for determining residues of carbadox in the 0.01–10 ppm range in swine feed is described. Carbadox is extracted from ground feed with 25% acidified methanol–$CHCl_3$, removed from emulsion-forming coextractables via an alumina column, separated from highly colored pigments by acid-base liquid-liquid partitioning, and finally isolated from interferences on a second alumina column. Isocratic reverse phase LC at 305 nm is used for quantitation. The average overall recovery at the 0.1, 0.5, and 1.0 ppm spike levels was 83.0% with a standard deviation of 2.04% and a coefficient of variation of 2.46%.

Carbadox (hydrazinecarboxylic acid, (2-quinoxalinylmethylene)-, methyl ester, *N'*, N^4-dioxide), trade name Mecadox, is a new animal drug used to increase the rate of weight gain, improve feed efficiency, and control swine dysentery and bacterial enteritis. Carbadox is a suspected carcinogen, and no residues are allowed in swine tissue (1). When using any of the available methodology (1–3), residue levels of carbadox/metabolite may be found in swine and other edible animal tissues as a result of misuse or accidental consumption of the drug.

To monitor and minimize cross-contamination of feeds with carbadox and therefore reduce the possibility of carbadox/metabolite residues in edible animal tissue, a method must be capable of reliably and selectively detecting carbadox in feeds down to levels of 0.01 ppm. In reviewing the literature, 1972 to present, only one method (4) was found for the determination of residue levels of carbadox in feeds. The method has a sensitivity of 24 ppb and the ability to separate carbadox from feed materials, other active drug ingredients, and possible carbadox decomposition products. This method appeared to satisfy the requirements of sensitivity and specificity but lacked consistency in recovery. As the level of carbadox decreased, it was necessary to increase the size of the feed sample to obtain the sensitivities reported. This caused the drug binding/matrix effect, which the author described, to be more pronounced.

The following liquid chromatographic (LC) procedure, which involved modification of certain medicated feed techniques (5, 6), in addition to having the sensitivity and specificity required, meets the requirements of reproducibility and reliability.

METHOD

Caution: Solutions of carbadox are light-sensitive. Protect both feed extracts and standard solutions from sun or artificial (fluorescent) light. Conduct analysis under conditions of diffused light. An example of an acceptable laboratory is one that is illuminated entirely by artificial light with the source of light at least 4 m from the work area.

Reagents

(a) *Solvents.*—Methanol and $CHCl_3$, distilled-in-glass (Burdick & Jackson Laboratories, Inc., Muskegon, MI).

(b) *N,N-Dimethylformamide (DMF).*—Certified ACS (Fisher Scientific Co.).

(c) *Acidified methanol.*—1% HCl–methanol.

(d) *Solution A.*—Acidified methanol—$CHCl_3$ (1 + 3).

(e) *Alumina.*—80-200 mesh (A-540, Fisher Scientific Co.).

(f) *Celite 545.*—Acid-washed (Johns Manville, Denver, CO).

(g) *Carbadox standard solutions.*—Store all standard solutions in low actinic glassware. *Stock solution.*—100 μg/mL. Accurately weigh 10.0 mg carbadox reference standard (Pfizer, Inc., Groton, CT) into 100 mL volumetric flask, dilute to volume with methanol–$CHCl_3$ (1 + 3), and mix well. *Working solution.*—1.0 μg/mL. Pipet 1.0 mL stock solution into 100 mL volumetric flask, dilute to volume with methanol–$CHCl_3$ (1 + 3), and mix well. *LC solution.*—1.0 μg/mL. Pipet 10.0 mL working solution into 100 mL pear-shape flask. Rotary-evaporate to dryness. Dissolve in 10.0 mL methanol–water (3 + 7).

(h) *Mobile phase.*—Methanol–water (35 + 65) at 40°C.

Apparatus

(a) *Chromatographic tubes.*—300 × 22 mm id (Kontes K-420300).

(b) *Chromatographic columns.*—400 × 22 mm id, fitted with coarse porosity fritted glass disks and Teflon stopcocks (Kontes K-420540).

(c) *Rotary evaporator.*—Rotavapor-R vacuum evaporator; water bath temperature 45–50°C (Brinkmann).

(d) *Receiving flasks.*—250 and 500 mL short-neck 24/40 glass-stopper joint, flat bottom (Corning No. 4100).

(e) *Acetylation flasks.*—100 mL pear-shape, short-neck 24/40 glass-stopper joint (Kontes K-608700).

(f) *Liquid chromatograph.*—Hewlett-Packard Model 1084B, equipped with variable wavelength detector, variable volume injector, and automatic sampling system. Operating conditions: chart speed 0.5 cm/min; attenuation 2 ↑ 3; zero offset 15%; mobile phase flow 1.0 mL/min; detector setting, sample 305 nm and reference 500 nm; column temperature ambient; column pressure 190 bar (2755 psi); volume injected 20 μL.

(g) *LC column.*—Resolve™ 15 cm × 3.9 mm id stainless steel, packed with 5 μm spherical C18 (P/N 85711, Waters Associates, Milford, MA).

(h) *Filter paper.*—Glass fiber, grade GF/A, 2.4 cm diameter (Whatman).

Table 1. Recovery of 0.01–10 ppm added carbadox

Day	Assay No.	Added, ppm	Rec., ppm	Rec., %
1	1	0.010	0.008	77.4
	2	0.052	0.046	88.2
	3	0.105	0.090	85.4
	4	1.05	0.871	83.0
	5	10.9	7.38	67.7
2[a]	1	0.010	0.007	67.6
	2	0.010	0.007	67.6
	3	0.105	0.083	79.3
	4	1.05	0.831	79.1
	5	10.9	7.837	71.9

[a]Final LC solution kept in refrigerator over weekend before injection on liquid chromatograph.

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Table 2. Recovery data on carbadox

	Assay No.	Added, ppm	Rec., ppm	Rec., %
Day 1	1	0.105	0.086	81.9
	2	0.105	0.085	81.0
	3	0.105	0.085	81.0
	4	0.105	0.088	83.8
	5	0.105	0.086	81.9
x, %				81.9
SD, %				1.143
CV, %				1.40
Day 2	1	0.524	0.448	85.5
	2	0.524	0.431	82.3
	3	0.524	0.422	80.5
	4	0.524	0.448	85.5
	5	0.524	0.422	80.5
	6	0.524	0.444	84.7
x, %				83.2
SD, %				2.375
CV, %				2.86
Day 3	1	1.05	0.910	86.7
	2	1.05	0.870	82.9
	3	1.05	0.843	80.3
	4	1.05	0.882	84.0
	5	1.05	0.882	84.0
	6	1.05	0.894	85.1
x, %				83.8
SD, %				2.156
CV, %				2.57
Overall				
x, %				83.0
SD, %				2.044
CV, %				2.46

Table 3. Recovery of carbadox from 2 types of feed

Feed	Assay No.	Added, ppm	Rec., ppm	Rec., %
Hog concentrate-40[a]	1	0.101	0.075	74.6
	2	0.101	0.079	78.6
	3	1.01	0.857	84.9
	4	1.01	0.831	82.3
Swine builder (A)[b]	1	0.101	0.083	82.2
	2	0.101	0.089	87.7
	3	1.01	0.952	94.3
	4	1.01	0.945	93.6

[a]High protein feed, 40% crude protein.
[b]High alfalfa-containing feed.

Extraction

Grind feed to pass 2 mm sieve. Weigh 50 g ground feed into 500 mL centrifuge bottle. Add 200.0 mL Solution A, stopper, and shake on mechanical shaker 30 min. Centrifuge 5 min at 1500 rpm. To chromatographic tube (a), with plug of glass wool and filter paper as support, add ca 8–10 g Celite followed by plug of glass wool. Prewet Celite with 50 mL Solution A. Quantitatively transfer 100 mL supernate to column, using 100 mL graduate cylinder. Rinse walls of graduate cylinder with 10 mL Solution A and add to column. Wash sides of column with 10 mL Solution A and let washing enter column bed. Wash graduate cylinder with 50 mL Solution A and add wash to column. Rinse tip of column with Solution A. Collect all eluates in 250 mL Phillips beaker. (Pressure may be applied with hand bulb to speed flow during this filtering step.)

Alumina Column Cleanup

To chromatographic column (b) add alumina to height of 15 cm followed by wad of glass wool. Add 50 mL Solution A to column and let drain. Discard eluate. Place 500 mL receiving flask under column. Transfer solution in 250 mL Phillips beaker to prepared column. Let solution enter column bed. Rinse walls of Phillips beaker with 10–20 mL Solution A and add rinse to column. Rinse walls of column with 10 mL Solution A. Wash Phillips beaker with 50 mL Solution A and transfer washing to column. Let column drain completely. With hand bulb, expel remaining solvent into receiving flask. Rotary-evaporate eluate just to dryness. Dissolve residue in 50 mL $CHCl_3$.

Acid-Base Extraction

Transfer 50 mL $CHCl_3$ extract in 500 mL receiving flask to 125 mL separatory funnel. Rinse receiving flask with 10 mL 1N NaOH and transfer to 125 mL separatory funnel. Stopper funnel and shake vigorously 30 s. Let phases separate (ca 5 min). Drain and discard lower $CHCl_3$ layer, keeping any emulsions. Wash receiving flask with 50 mL $CHCl_3$ and transfer wash to separatory funnel. Stopper funnel and shake vigorously 30 s. Let phases separate (ca 5 min). Drain and discard lower $CHCl_3$ layer, keeping any emulsions. Add 12 mL 1N HCl to separatory funnel. Rinse 500 mL receiving flask with 50 mL $CHCl_3$ and add rinse to aqueous layer in 125 mL separatory funnel. Stopper and shake vigorously 30 s. Let phases separate (ca 5 min). Drain lower $CHCl_3$ layer into 250 mL receiving flask via 35–40 g Na_2SO_4. Repeat rinsing and extracting with two 50 mL portions of $CHCl_3$. Rotary-evaporate combined $CHCl_3$ extracts to dryness. Dissolve residue in 5 mL DMF and proceed to *Alumina Column Isolation*.

Alumina Column Isolation

To chromatographic tube (a), with plug of glass wool partially inserted into stem followed by filter paper as support, add alumina to height of 3 cm followed by wad of glass wool. Prewet column with 25 mL DMF. Transfer DMF solution from 250 mL receiving flask to column. Complete transfer with 2 more 5 mL portions of DMF. Wash column with three 10 mL portions of DMF. Wash column with three 25 mL portions of $CHCl_3$, rinsing 250 mL receiving flask each time with $CHCl_3$ washes before adding to column. Discard all eluates to this point. Place 100 mL pear-shape flask under

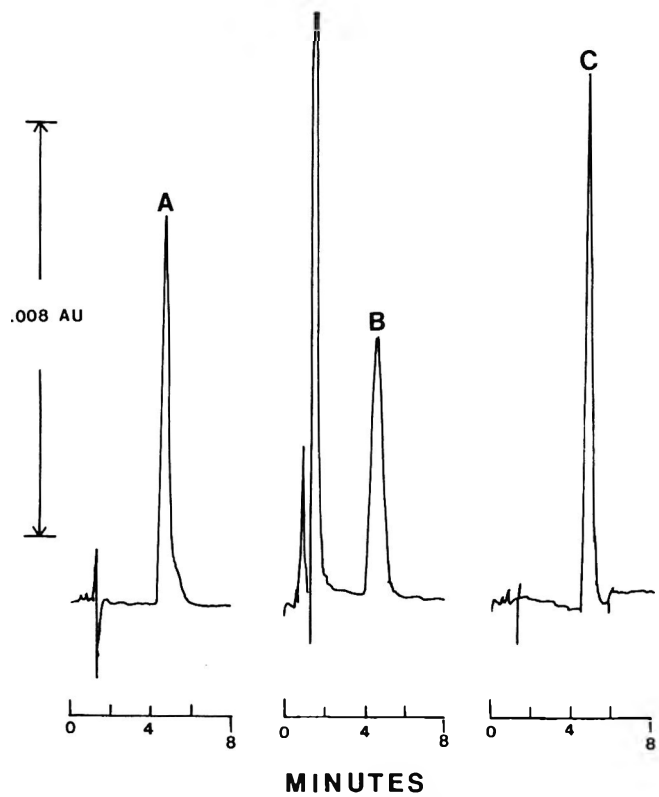

Figure 1. Chromatograms of carbadox (1.05 μg/mL) prepared in different solvents. A, methanol; B, DMF; C, 30% methanol–water. In each case 20 μL (21 ng) was injected.

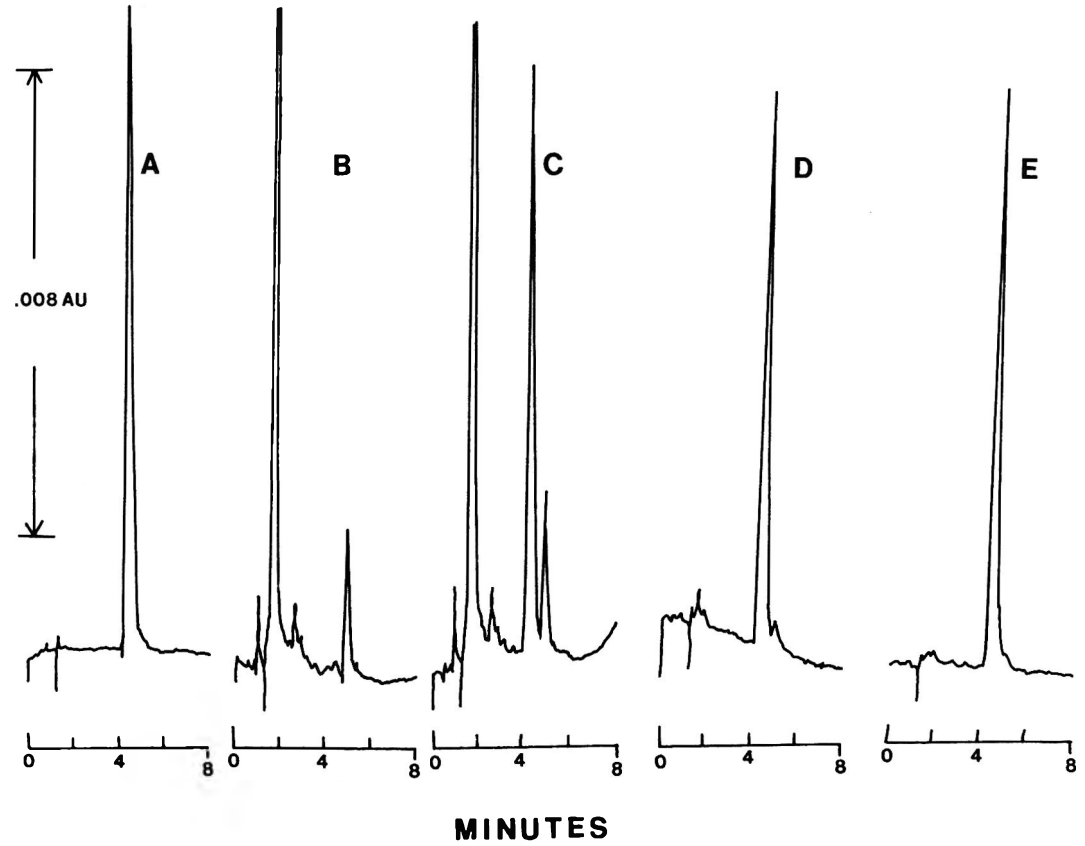

Figure 2. Chromatograms of A, carbadox standard (1.05 μg/mL, 20 μL injected (21 ng)); B, unspiked feed (10 g/mL); C, D, and E, feed spiked with 0.1, 0.5, and 1.0 ppm carbadox, respectively.

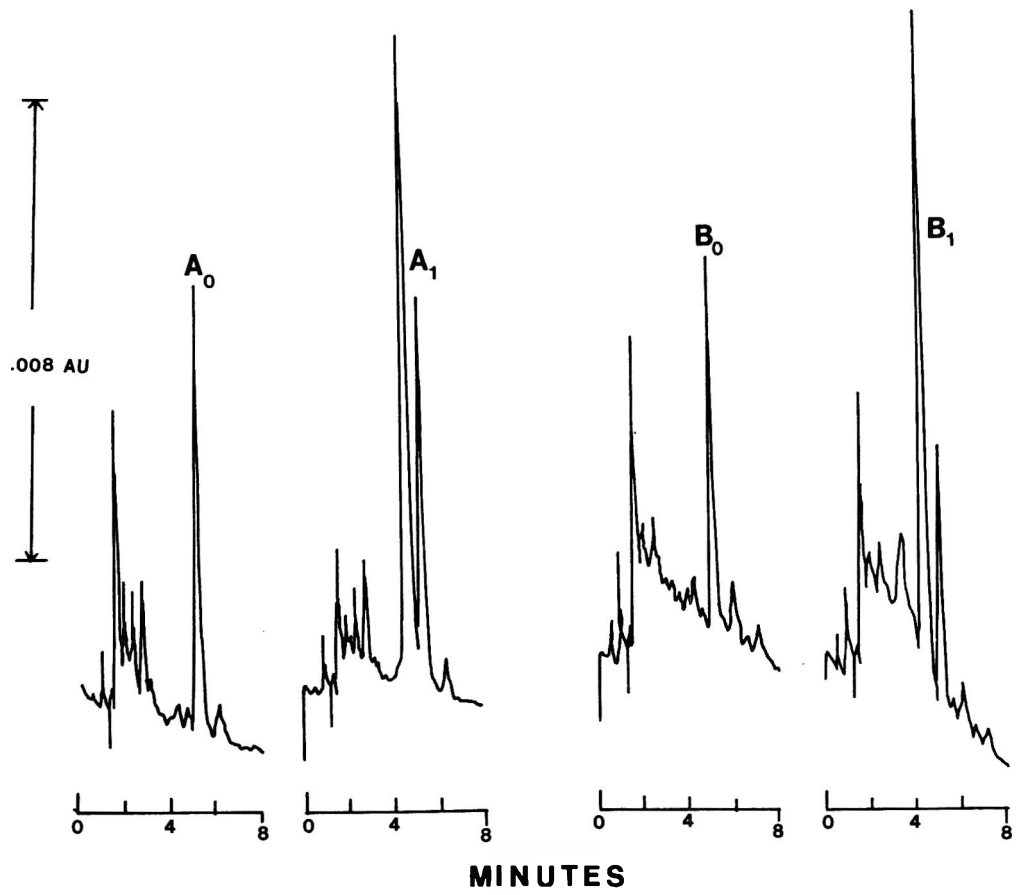

Figure 3. Chromatograms of A_0, unspiked hog concentrate-40 (12.5 g/mL); A_1, spiked hog concentrate-40 (0.1 ppm); B_0, unspiked swine builder (A) (12.5 g/mL); B_1, spiked swine builder (A) (0.1 ppm).

column. Elute carbadox with 60 mL methanol–$CHCl_3$ (1 + 3). Let column drain completely. With hand bulb expel remaining solvent into flask. Rinse tip with methanol.

Liquid Chromatography

Rotary-evaporate methanol–$CHCl_3$ solution containing carbadox just to dryness. Dissolve residue in appropriate volume (2.0 mL) of 30% methanol–water to obtain concentration of ca 1 μg carbadox/mL. Inject 20 μL sample and LC carbadox standard solutions into liquid chromatograph. Bracket sample with injections of carbadox standard solution.

Calculation

$$\text{Carbadox, ppm} = (Asam/Astd) \times (C/W) \times D \times (Vstd/Vsam)$$

where $Asam$ and $Astd$ = peak area of sample and standard, respectively; C = concentration of carbadox, μg/mL; W = sample weight, g; D = dilution of sample, mL; $Vstd$ and $Vsam$ = volume of standard and sample injection, respectively.

Results and Discussion

Recovery of carbadox from feeds was performed on a nonmedicated, locally produced, commercial hog finishing feed. This feed was spiked by adding a known amount of carbadox standard solution to a clean 500 mL centrifuge bottle and allowing it to air dry. Feed was weighed into the centrifuge bottle, mixed, and analyzed as described in the method. Wet spiking of feed was avoided to prevent carbadox-solvent-feed matrix interaction.

Single rather than multiple extraction was chosen because multiple extractions, in addition to being more time consuming, did not improve recovery and increased unwanted excipients.

Thorpe (5) reported that carbadox medicated feeds in pellet form generally required a water pretreatment to release the drug absorbed on feed ingredients during the pelleting process. Luchtefeld (4) reported that apparent drug binding to feed material became more pronounced at increased levels of feed material and decreased drug levels. We also found this to be the case. When the 1.0 ppm spike was added only to the supernate, the recovery was 94.7%; when the spike was added to the extracting solvent and feed, recovery was 74.5%; and when it was added directly to the feed, recovery was 68.6%. Thus the carbadox was not being bound by the coextractables, but rather by the actual feed matrix, which was functioning as an absorbent. This interaction between feed matrix and carbadox becomes significant when performing drug residue determinations. An extraction system that would extract carbadox from feed efficiently and quantitatively while being compatible with the previously reported method (7) was investigated. Several solvents and solvent combinations were tried without success.

Since we knew that *(1)* carbadox would be extracted into $CHCl_3$ from an acid-buffered media, *(2)* carbadox is insoluble in water, and *(3)* the interaction between carbadox and the feed matrix was probably due to H-bonding, acidification of the extracting solvent seemed a logical approach. This was accomplished by combining the acid with methanol. The acidified methanol was then used to prepare the extracting solvent, 25% methanol–$CHCl_3$. This step would require the removal of any acid or water before addition to the alumina column, which was accomplished by extracting the $CHCl_3$ with water to remove the acid and by filtering the $CHCl_3$ through anhydrous Na_2SO_4 to remove the water.

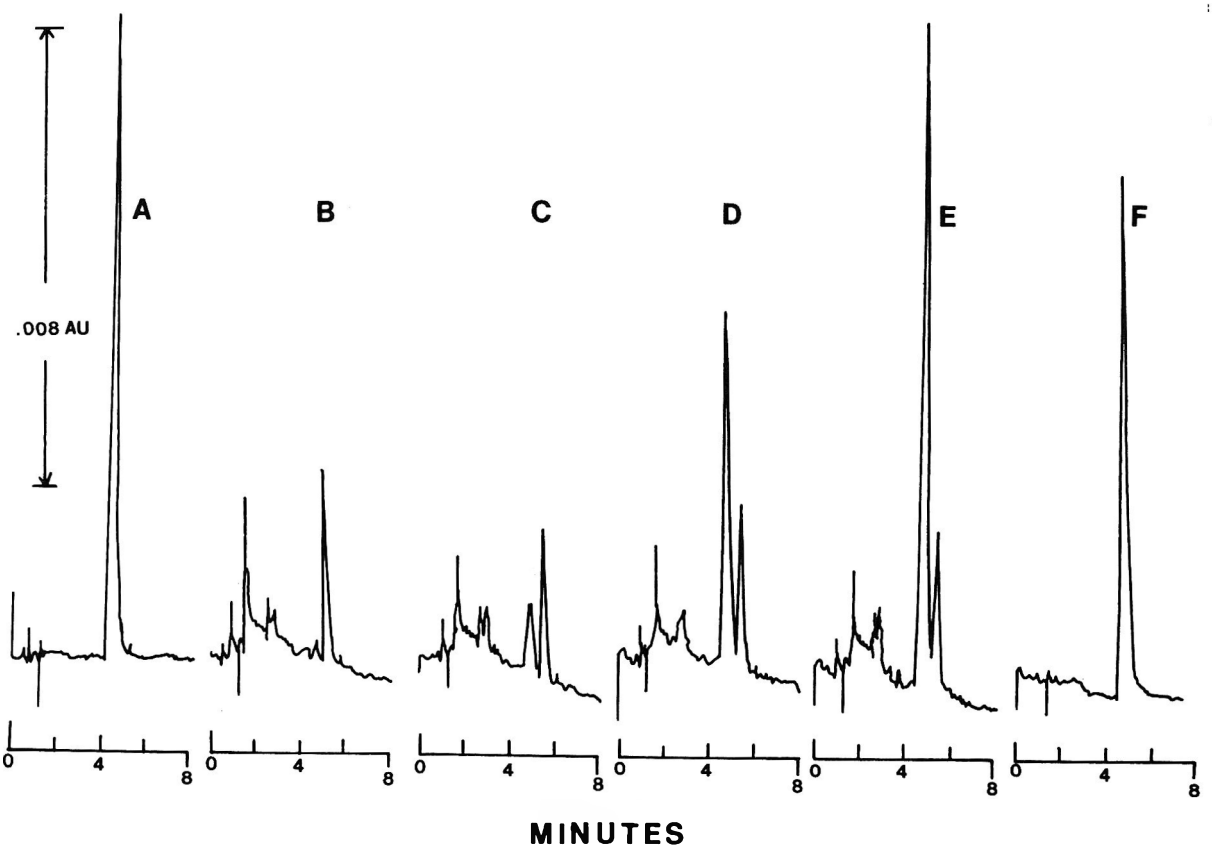

Figure 4. Chromatograms of A, carbadox standard (1.05 μg/mL, 20 μL injected (21 ng)); B, unspiked feed (12.5 g/mL); C, D, E, and F, feed spiked with 0.01, 0.05, 0.1, and 1.0 ppm carbadox, respectively.

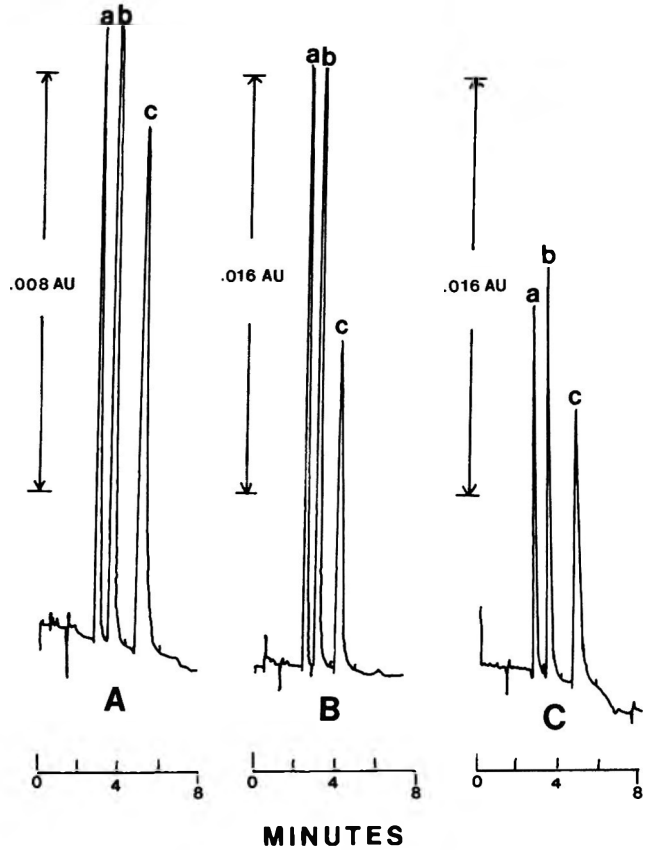

Figure 5. Chromatograms showing separation of carbadox from other veterinary drugs. A, a, nitrofurazone (5.1 μg/mL); b, furazolidone (7.5 μg/mL); c, carbadox (1.05 μg/mL). Attenuation 2 ↑ 3. B, a, nitrofurazone (10.2 μg/mL); b, furazolidone (15.0 μg/mL); c, carbadox (1.05 μg/mL). Attenuation 2 ↑ 4. C, same as A except attenuation 2 ↑ 4.

Several acids, acetic, citric, sulfuric, formic, nitric, and hydrochloric, as well as water, were used as 1% solutions in methanol. Except for acetic acid, all produced recoveries ≥70%. Hydrochloric acid gave the best recovery, 81.9%. Different percentages of hydrochloric acid, up to 10%, in methanol were then tried. As the percentage of hydrochloric acid was increased, the contribution to release of carbadox from the feed matrix was decreased due to either the formation of an amine salt, which is soluble in water and was lost in the initial water wash, or the hydrolysis to the carboxaldehyde with a resulting change in solubility, UV absorbance, and/or retention time. The method was later modified to eliminate the initial water wash and include the first alumina column cleanup step.

The average partition coefficient of carbadox into $CHCl_3$ from an acidified aqueous solution was 0.89, and this was sufficient to recover 99% of the carbadox with three 50 mL $CHCl_3$ extractions. Table 1 shows the range at which the proposed method was tested, 0.01–10 ppm, and the recoveries observed. Recoveries of carbadox are shown in Table 2; day-to-day as well as overall variations are noted. The recovery of carbadox from feeds containing large amounts of alfalfa and protein is shown in Table 3.

Figure 1 shows the effect of using different solvents for the final dilution for LC. Although the peak shapes and heights varied depending on which solvent was used, the total peak areas and retention times (RT) remained relatively constant: A = 15 740 (RT 4.69 min), B = 15 430 (RT 4.61 min), and C = 15 760 (RT 4.73 min). Therefore, we can choose the final solvent for LC that is more compatible with the sample matrix encountered without adversely affecting the calculation when using peak areas for quantitation.

The solvent of choice was 30% methanol–water because it not only gave better peak shape and height for quantitation by either area or height, but also allowed greater sensitivity of detection. The solubility of carbadox in DMF is greater than in 30% methanol–water; however, the problem of limited solubility can be easily overcome by increasing the mixing time of the solvent with the residue. DMF also solubilized unwanted materials, which might tend to affect the column with time.

Figure 2 shows typical LC chromatograms of control feed and feed spiked with carbadox at different levels. The LC system resolves the analyte from background interferences at the 0.1 ppm level; background no longer is an influencing factor at ≥0.5 ppm levels. No attempt was made to identify the peak in the control feed, which has a relative retention time (RRT) of 1.15 with respect to carbadox. The background that may be encountered in feeds with high protein or high alfalfa content at the 0.1 ppm level is shown in Figure 3. The only dominant peak (RRT 1.15) in the control feed is well resolved from carbadox for quantitation. Typical LC chromatograms showing the range of the proposed method are in Figure 4, which indicates the obvious resolution and sensitivity of the procedure. Under the LC conditions described, separation of widely used veterinary drugs that are likely to be encountered in swine feeds is possible with baseline resolution, as shown in Figure 5. Figure 5 shows that carbadox is still well resolved for quantitation in the presence of nitrofurazone and furazolidone at concentrations of 10 and 15 times greater than carbadox, respectively. Feeds were spiked with these 2 drugs and it was found that they were removed before the LC step. Another drug, pyrantel tartrate (ultraviolet maximum 312 nm), commonly found in combination with carbadox, does not elute under these LC conditions but is retained on the column. It may be removed by eluting with a higher percentage of methanol (80%).

The results indicate that the proposed method is acceptable for routine determination of carbadox residues in animal feeds. However, because of the large variations in feed composition, it is suggested that duplicate spiked feed samples be included for each type of feed being analyzed to determine the effect of that particular feed on carbadox recovery.

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Liquid Chromatographic Determination of Carbadox and Desoxycarbadox in Medicated Feeds and in Porcine Gastrointestinal Tract

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A liquid chromatographic method for the assay of carbadox and desoxycarbadox in medicated feeds and porcine stomach and intestinal contents is described. Samples were extracted with dimethylformamide–water and cleaned up on an alumina column. The eluate was chromatographed using either gradient elution for simultaneous assay of both compounds or isocratic elution for carbadox only. Detection of carbadox by its native fluorescence yielded a sensitive and specific assay without interferences by metabolites or matrix components. The optimal UV absorption of desoxycarbadox was at 280 nm. Mean recoveries of carbadox in spiked feed and stomach contents were 104 and 97%, respectively; mean recovery of desoxycarbadox in stomach contents was 106%. The day-to-day reproducibility for carbadox in different feed samples and stomach contents samples had a coefficient of variation of 6–13%.

Carbadox (methyl-3-(2-quinoxalinylmethylene) carbazate-N^1,N^4-dioxide) is used in swine feed as a growth-promoting substance to improve feed efficiency. It is also used as a prophylacticum against swine dysentery (*Treponema hyodysenteriae*). In feeding experiments to study toxicological and pharmacological properties of carbadox in pigs, a sensitive and reproducible assay was needed for carbadox and one of its metabolites, desoxycarbadox (methyl-3-(2-quinoxalinylmethylene) carbazate), in feed, and in stomach and intestinal contents. These experiments will be reported elsewhere.

Previously published methods described the determination of carbadox using spectrophotometry (1, 2) or liquid chromatography with UV detection (3–7). Gas chromatography/mass spectrometry was used for confirmatory identification of carbadox-related residues in swine liver (8). The procedure presented here provides a more sensitive determination of carbadox in feed, and can also be applied to stomach and intestinal contents, using liquid chromatography with fluorescence detection. In addition, a gradient elution program was developed for simultaneous assay of carbadox and desoxycarbadox in stomach and intestine contents, with UV detection.

METHOD

Apparatus and Reagents

(a) *Liquid chromatograph.*—Spectra-Physics Model SP 8700 with SP 4100 computing integrator, and detectors connected in series. UV detection (Spectra-Physics Model SP 8400) of desoxycarbadox at 280 nm at 0.01 absorbance unit full scale; fluorescence detection (Perkin-Elmer fluorescence spectrophotometer Model PE 3000) for carbadox at excitation 310 nm and emission 487 nm.

(b) *Chromatographic column.*—Chrompack 10RP18 or Chrompack CP Spher C_{18}, 25 cm × 4.6 mm id, connected with a guard column (10 cm × 4.6 mm id) containing pellicular reverse phase packing.

(c) *Mobile phase.*—For carbadox assay in absence of desoxycarbadox, an isocratic mixture of acetonitrile–water (16.5 + 83.5) was used at a flow rate of 1.5 mL/min. For simultaneous assay of carbadox and desoxycarbadox, a gradient elution program was used. Starting at acetonitrile–water (10 + 90) until t = 1 min, composition was linearly programmed to (50 + 50) at t = 11 min, holding this composition until t = 12 min. Flow rate was 1.5 mL/min. Solvents used were acetonitrile LiChrosolv (Merck) and LC-grade water (J. T. Baker Chemicals).

(d) *Sample extraction solvent.*—Mixture of *N,N*-dimethylformamide (pro analyse, Merck) and LC-grade water (J. T. Baker Chemicals) (95 + 5).

(e) *Cleanup column.*—5 g basic aluminum oxide, 70–230 mesh, activity I (Merck, Art No. 1076) in cotton-plugged 8 mm id chromatographic tubes.

(f) *Stock standard solutions.*—Accurately weigh ca 25 mg carbadox (Pfizer B.V., Rotterdam, Lot 194-15) and ca 50 mg desoxycarbadox (Pfizer B.V., Rotterdam, Lot 8735-171-1) into separate 100 mL volumetric flasks. Dilute to volume with extraction solvent. Use low actinic glassware and protect from light.

(g) *Working standards.*—Dilute stock standard solutions with extraction solvent to final concentrations of 0.25 μg/mL of carbadox and 0.5 μg/mL of desoxycarbadox. Use low actinic glassware and protect from light.

Preparation of Sample Extract

Freeze-dry stomach and intestinal content samples. Grind dried samples and feed samples in mortar and accurately weigh 0.1–1 g sample, depending on expected concentration, into 100 mL glass-stopper Erlenmeyer flask.

Add 10 mL extraction solvent and swirl on shaking machine 2 h; let stand overnight. Proceed with extraction and cleanup according to Thorpe's method (4). Inject 50 μL of cleaned extract onto liquid chromatographic column.

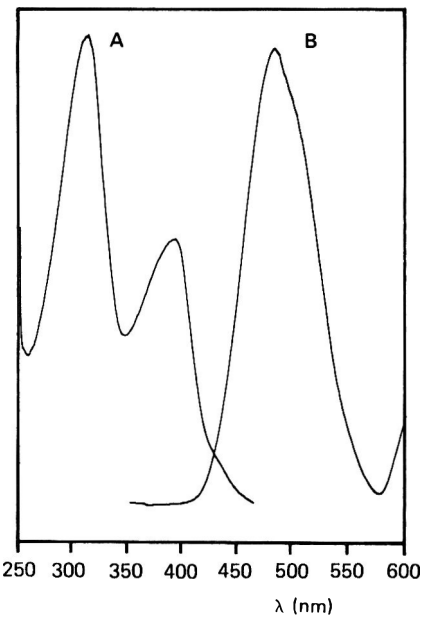

Figure 1. Fluorescence spectra of carbadox, 10 μg/mL in acetonitrile–water (50 + 50). A, Excitation spectrum, 487 nm. B, Emission spectrum, 310 nm. Slit width excitation and emission, 15 and 5 nm, respectively. Scan speed 30 nm/min.

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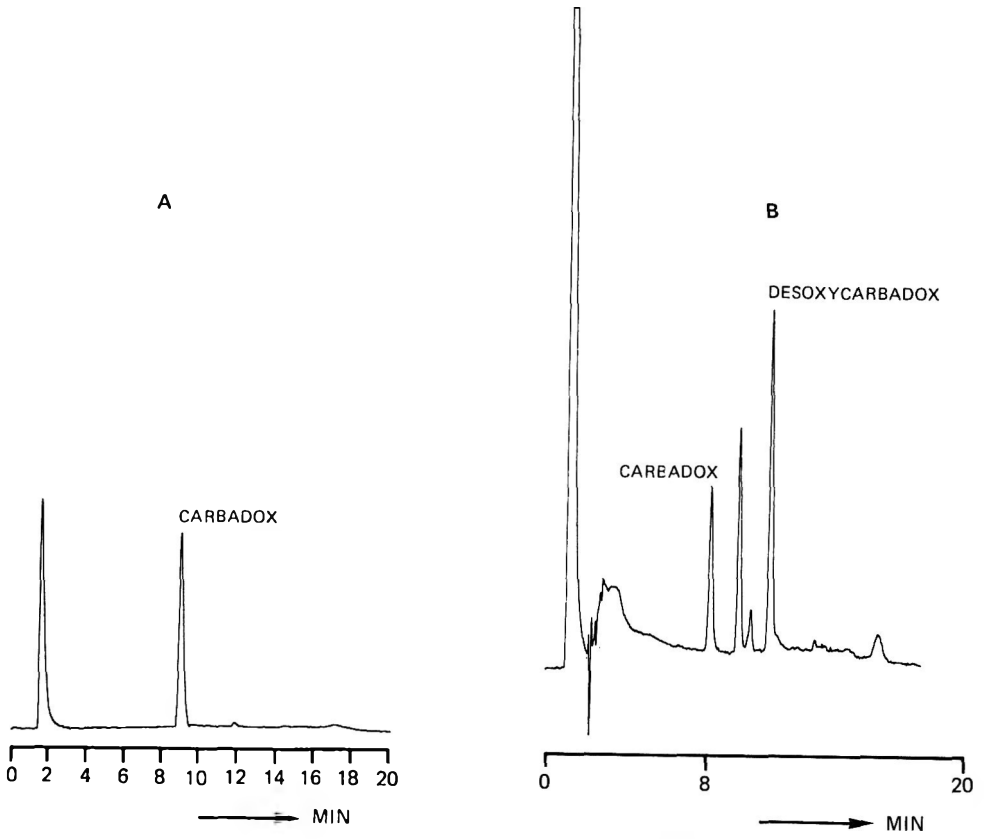

Figure 2. Liquid chromatography of duodenum contents of carbadox-fed swine; Chrompack CP Spher C_{18} column, gradient elution acetonitrile–water. A, Fluorescence detection, 310 nm/487 nm. B, UV detection, 280 nm.

Results and Discussion

Preliminary experiments showed that carbadox exhibits native fluorescence; Figure 1 presents the fluorescence excitation and emission spectra. In this study, the fluorescence phenomenon was used to develop a liquid chromatographic analysis of carbadox.

An example that shows the advantages of fluorescence detection compared with UV detection is given in Figure 2. A sample of duodenum contents of a pig fed carbadox was extracted and cleaned as described. The cleaned extract was chromatographed twice under identical chromatographic conditions (gradient elution) using UV detection (Figure 2A) or fluorescence detection (Figure 2B). The UV tracing shows a less stable baseline, while desoxycarbadox and other unidentified metabolites are detected. When amounts of carbadox metabolites are not of interest (e.g., feed samples or pharmacokinetic studies in experimental animals), fluorescence detection is the method of choice because of its selectivity and its almost 2-fold gain in sensitivity compared with UV detection at 305 nm. If a simultaneous assay of carbadox and desoxycarbadox is preferred, the optimal wavelength for UV detection is 280 nm for desoxycarbadox or 305 nm for carbadox.

Minimum detectable amounts of carbadox with fluorescence detection are about 0.1 ng. The corresponding minimal concentration of carbadox detectable in a 1 g sample according to this method is about 20 ppb. For desoxycarbadox, these quantities for a 1 g sample are 0.2 ng and 40 ppb, respectively. Response for carbadox and desoxycarbadox was linear in the range 2.5–100 ng.

In a series of experiments, feed samples were spiked with carbadox and swine stomach contents were spiked with carbadox and desoxycarbadox to determine recoveries. Carbadox was assayed using fluorescence detection, desoxycarbadox using UV detection at 280 nm. Because of the relatively high sensitivity of the detection, it was possible to perform recovery experiments with small amounts of matrix material (0.1–1 g), spiked at different concentrations. This minimized matrix effects and effects of interfering compounds. Results are presented in Table 1. The day-to-day reproducibility of the carbadox assay by fluorescence detection was tested by analyzing 3 feed samples and 3 swine stomach contents samples 13 times during a 46-day period. All carbadox concentrations were unknown at the time of analysis. The results are presented in Table 2 and show about 10% variation over a 100-fold concentration range and an almost 7-week period. This indicates a reliable assay.

Table 1. Mean recoveries of carbadox and desoxycarbadox in spiked feed samples and swine stomach contents samples

Assay	Range of concn, mg/kg	Mean rec., %	SD	Analyses
Carbadox in feed	1–200	104	7	40
Carbadox in stomach	1–50	97	8	13
Desoxycarbadox in stomach	1–50	106	10	23

Table 2. Means, standard deviations (mg/kg), and coefficients of variation (%) of 13 carbadox assays in 3 feed samples (A to C) and swine stomach contents samples (D to E) during 46-day period

	Feed			Stomach		
Statistic	A	B	C	D	E	F
Mean	1.15	73	128	1.09	77	108
SD	0.12	5.6	9.0	0.07	8.4	13.9
CV	10.4	7.6	7.0	6.1	11.0	12.9

In conclusion, this method permits the quantitative determination of carbadox and desoxycarbadox in biological material. It is more sensitive and selective than previously published methods. Interfering compounds were not observed, and reproducibility is quite satisfactory.

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DRUG RESIDUES IN ANIMAL TISSUES

Sensitive Enzyme Immunoassay of Colistin and Its Application to Detect Residual Colistin in Rainbow Trout Tissue

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An antibody against colistin (CL), an antibiotic effective for gram-negative bacteria, was produced in rabbits immunized with a colistin-protein conjugate. The conjugate was prepared by a novel and convenient procedure devised to couple an amino group of CL to thiol groups of bovine serum albumin (BSA) introduced by thiol exchange reduction of its disulfide bonds with dithiothreitol, using *N*-(*m*-maleimidobenzoyloxy)succinimide (MBS) as a cross-linker. Enzyme labeling of CL with β-D-galactosidase was performed by utilizing another cross-linker, *N*-(γ-maleimidobutyryloxy)succinimide, by means of a convenient labeling method. A double antibody enzyme immunoassay of CL, which could determine as little as 30 ng/mL of CL, was developed using labeled CL and anti-CL antiserum. With this assay, drug levels were easily determined in fish tissue after CL administration. The enzyme immunoassay should provide a useful tool for detection and quantitation of residual drugs in foods and related products.

Veterinary drug residues in food-producing animals are of great concern because of their health hazard potentials. Methods for detecting residual drugs in food products of animal origin have been studied extensively (1). Still, no completely acceptable method is available for use under industrial conditions. The usual analytical methods for drug detection require time-consuming separation procedures that are impractical.

Recent development of a variety of immunological assays, especially enzyme immunoassay (EIA) (2–5), has made possible highly sensitive, specific, precise, rapid, and convenient measurement of drug concentrations. We previously introduced a new method for selectively modifying proteins by using hetero-bifunctional reagents of a maleimide succinimidyl ester type (6–8), and applied the reagent in a new enzyme-labeling method of antigens or antibodies (7–10) and also in a new method for preparing hapten immunogens (11–14). Using the 2 methods in studies on colistin (CL), a peptide antibiotic that is widely used in Japan as a veterinary drug (15–17), we were able to prepare an enzyme-labeled CL and a rabbit anti-CL antiserum. With these 2 preparations as immunoreagents, we developed a highly sensitive enzyme immunoassay (EIA) for CL. The present paper reports on these studies together with application of EIA of CL for easy detection of residual CL in fish tissue.

METHODS

Materials

(a) *β-D-Galactosidase (Gal).*—From *Escherichia coli* (EC 3.2.1.23). Reagent grade for EIA (Boehringer Mannheim Biochemicals, Indianapolis, IN 46250).

(b) *Antibiotics.*—Commercial products: cephalexin (Shionogi Pharmaceutical Ind., Osaka, Japan), ampicillin (Takeda Chemical Ind., Osaka, Japan), dihydrostreptomycin (Kaken Kagaku Co., Tokyo, Japan), and colistin (Meiji Seika Co., Tokyo, Japan).

(c) *Cross-linkers.*—*N*-(*m*-Maleimidobenzoyloxy)succinimide (MBS) and *N*-(γ-maleimidobutyryloxy)succinimide (GMBS) were prepared by published methods (7, 10, 18).

(d) *Reagents.*—Other reagents were all reagent grade.

Assay Media

Buffer A: 0.02M sodium phosphate buffer, pH 7.0, containing 0.1M NaCl, 1mM $MgCl_2$, 0.1% bovine serum albumin (BSA), and 0.1% NaN_3. Buffer B: phosphate–EDTA buffer, pH 7.4 (0.03M sodium phosphate dibasic–1mM EDTA disodium salt, containing 0.1% BSA).

Preparation of Immunogen for CL

A 3-step synthesis was used as shown in Figure 1.

Step 1: MBS-acylated colistin.—A solution of CL (7 μmol) in 0.05M sodium phosphate buffer (pH 7.0, 3 mL) was incubated 30 min at 30°C with tetrahydrofuran solution of MBS (2.2 mg/0.3 mL, 7 μmol), with occasional stirring. Tetrahydrofuran was removed by flushing with nitrogen, and excess MBS was extracted from the reaction mixture with three 5 mL portions of methylene chloride. The aqueous layer was used for step 3.

Step 2: Reductive cleavage of disulfide bonds in BSA.—A solution of BSA (0.1 μmol) in 2 mL 0.2M tris–hydrochloride buffer (pH 8.6) containing 8M urea was incubated with 1.54 mg dithiothreitol (DTT) (10 μmol) 1 h at 30°C. The reduced BSA was precipitated by adding 3 mL 10% trichloroacetic acid. After washing twice with 1 mL water, the precipitate was dissolved in 0.02M phosphate-buffered 6M urea and the solution was used for the next step.

Step 3: Conjugation of MBS-acylated colistin to reduced BSA.—The solution of reduced BSA (step 2) was incubated 2 h at 25°C with the aqueous layer of MBS-acylated colistin

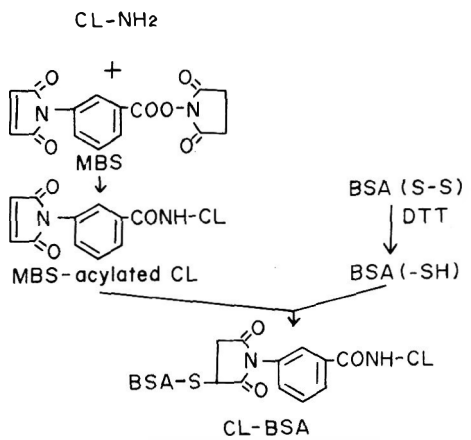

Figure 1. Scheme for preparing CL immunogen by using a hetero-bifunctional cross-linker, MBS: CL–NH_2, colistin; MBS, *N*-(*m*-maleimidobenzoyloxy)succinimide; DTT, dithiothreitol; BSA, bovine serum albumin.

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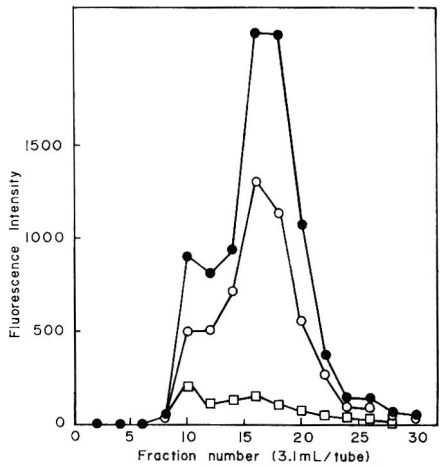

Figure 2. Elution profiles of CL–Gal from a Sepharose CL 6B column. Enzyme activity of conjugate (closed circles) measured with 5 μL of each fraction for 5 min at 30°C; immunoreactive enzyme activity (open circles) determined by EIA as described except that 5 μL conjugate and a 1:1000 solution of anti-CL–MBS–BSA antiserum were used in absence of CL; immunoreactive enzyme (open squares) determined in competition with 50 μg CL in same manner.

(step 1), and the mixture was chromatographed on a 2.8 × 48 cm Sephadex G-100 column with 3M urea as eluant. The conjugate fractions were pooled and stored at 4°C until used for immunization.

Immunization

Two female New Zealand white rabbits were injected subcutaneously and intramuscularly with 1.3 mL conjugate solution (ca 1.0 mg protein) emulsified in an equal volume of complete Freund's adjuvant. Two booster injections of half the first dose were given at monthly intervals. The rabbits were bled from the ear veins before immunization and 2 weeks after each injection. The sera were stored at −30°C.

Preparation of Colistin–β-D-Galactosidase (CL–Gal) Conjugate

A tetrahydrofuran solution of GMBS (0.1 μmol/30 μL) was added dropwise to a solution of 1.2 mg CL in 1 mL 0.02M sodium phosphate buffer (pH 7.0), with vortex-mixing, and was incubated 30 min at 30°C. The mixture was then added dropwise to a solution of Gal (50 μg, 93 pmol in 1 mL) in the same buffer and the mixture was incubated 2 h at 30°C. The solution was loaded onto a 1.4 × 43 cm Sepharose CL-6B column and eluted with buffer A. The enzyme and immune activity of 5 μL of each fraction (3.1 mL/tube) were measured as described below.

Measurement of Enzyme Activity

Five μL diluted enzyme solution was incubated 30 min at 30°C with 0.15 mL 0.1mM 7-β-D-galactopyranosyloxy-4-methylcoumarin in buffer A. The reaction was stopped by the addition of 2.5 mL 0.2M glycin–NaOH buffer, pH 10.3, and the 7-hydroxy-4-methylcoumarin liberated was measured by spectrofluorometry. The amount of CL–Gal was expressed in units of Gal activity, and 1 U of enzyme activity was defined as the amount that hydrolyzes 1 μmol substrate/min.

Enzyme Immunoassay (EIA)

Buffer B was used as the dilution and incubation medium for EIA unless otherwise stated. The assay was performed by the double antibody method. CL–Gal (50 μU) and a known amount of CL or a sample were incubated 3 h at 25°C with 50 μL of 100 000-fold diluted solution of anti-CL–MBS–BSA antiserum in a final assay volume of 250 μL. Then 50 μL 0.25% solution of normal rabbit serum and 50 μL 5% solution of goat anti-rabbit IgG were added. After further incubation overnight at 25°C, 1 mL buffer A was added and the mixture was centrifuged at 4°C for 15 min at 2500 rpm. The supernatant fraction was removed and the enzyme activity in the immune precipitate was measured.

Measurement of Tissue Levels

A solution of CL (1 mg/0.5 mL) was injected into the efferent branchial artery of rainbow trout (weight 110 ± 20 g). The fish was killed by decapitation 2 h later. The organs were removed and homogenated in 5 volumes of ice-cold 5% trichloroacetic acid (19) using a Polytron Model PT 10 (Brinkmann Instruments, Westbury, NY). The homogenates were centrifuged at 4°C for 15 min at 2500 rpm and the supernates were collected and adjusted to pH 7.2 with 1.0N NaOH, and were used to quantitate the CL by EIA. Control samples of tissues and body fluids obtained from rainbow trout injected with saline were processed in the same manner.

Results

Preparation of CL–MBS–BSA Conjugate (CL Immunogen)

Preparation of the CL immunogen, with the formation of covalent bonds between the amino groups of CL and the thiol groups of chemically modified BSA, was achieved using the heterobifunctional cross-linker, GMBS (Figure 1). CL–MBS–BSA conjugate was isolated by chromatography on a Sephadex G-100 column.

Enzyme Labeling of CL

CL was labeled with Gal according to our earlier method (13), using the limited molar amount of a hetero-bifunctional reagent, GMBS. The GMBS-acylated CL formed in the reaction mixture was used directly for enzyme labeling of CL without processing to separate unreacted GMBS. No reduction in the enzyme activity was observed during the enzyme labeling process. The preparation was chromatographed on a Sepharose CL-6B column to remove any free CL remaining. The peak fraction of enzyme activity was parallel with that of immune specificity as judged by the enzyme immunoassay of CL (Figure 2).

Antibody Response

Antibodies against CL were produced in 2 rabbits immunized with the CL–MBS–BSA conjugate and were detected by the

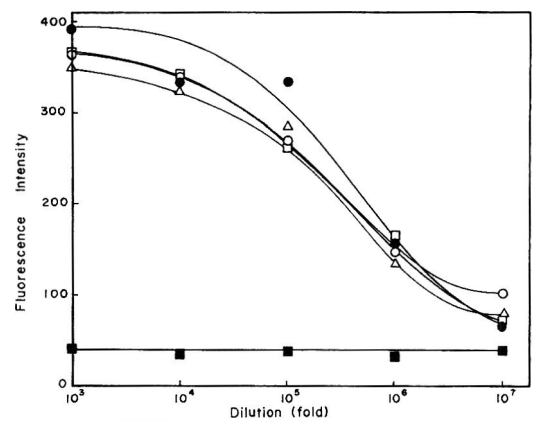

Figure 3. Qualitative estimation of anti-CL antibody. Samples 1 (closed circles), 2 (open squares), 3 (open circles), and 4 (open triangles) are antisera collected 1 month, and 2, 4, and 6 weeks, respectively, after first injection. NRS sample (closed squares) obtained from rabbit bled before immunization.

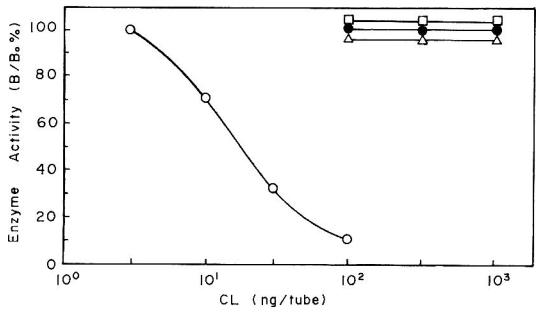

Figure 4. Standard curves for EIA of CL (open circles) and cross-reactivities with ampicillin (closed circles), cephalexin (open triangles), and streptomycin (open squares). Curve shows amount (percentage) of bound enzyme activity for various doses of CL (B) (3–100 ng) and 3 antibiotics with respect to that bound using CL–Gal alone (Bo).

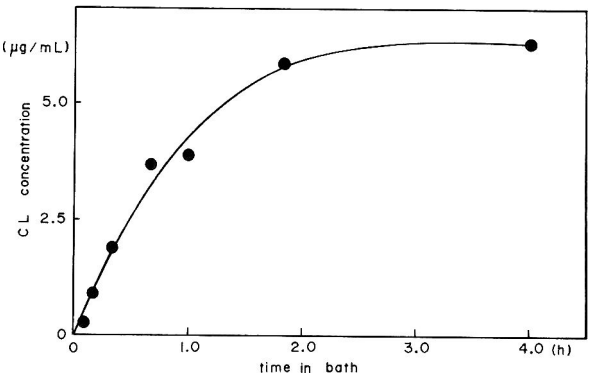

Figure 5. Serum CL levels in rainbow trout kept in CL bath (200 μg/mL). Blood was collected from 6 fish, weighing about 110 g each, and the mean of serum CL contents was estimated.

reaction of the diluted antiserum with the CL–Gal conjugate. The uptake of CL-Gal conjugate by the precipitate gave a quantitative measure of the bound antibody. Figure 3 shows typical binding curves for the sera from one of the 2 rabbits; the curves were obtained by using EIA procedure described without the competition of CL. The antibody titer in the serum against CL–Gal reached maximum 1 month after the first injection and was not affected by the booster injections. The titer was excellent and a 10 000 000-fold diluted solution of the antiserum could bind with CL–Gal, showing about a 2-fold enzyme activity value of that of the blank.

Enzyme Immunoassay

Inhibition of the binding of the CL–Gal conjugate to anti-CL–MBS–BSA antiserum by CL is shown in Figure 4. Addition of increasing amounts of the drug resulted in a progressive decrease of the conjugate precipitated.

Precision studies on the method are summarized in Table 1. Good recoveries (100.0–102.4%) were obtained for 4 CL samples, with coefficients of variation of less than 17.8 and 30.4% for within-day and between-day assays, respectively, with an overall mean of 19.1% (Table 1). CL in rainbow trout serum samples (50 μL) was also measured after known concentrations were added (Table 1); the standard curve was essentially the same as that obtained from the buffer system described above.

Antibody Specificity

The specificity of the anti-CL antiserum against CL was examined by cross-reaction studies applying EIA of CL. Antibiotics such as dihydrostreptomycin, ampicillin, and cephalexin were examined by EIA and none showed significant inhibition (Figure 4).

Experiments with a microbiological assay using *Escherichia coli* NIHJ were done to compare sensitivity for that method of CL detection with that of the EIA. Results showed that the maximal sensitivity of the microbiological assay, using 20 μL CL solution in a paper disk method, allowed detection of 10 μg/mL. This was 333 times less sensitive than the EIA (0.03 μg/mL).

Measurement of Tissue CL Levels

CL was administered i.a. to fish, and the drug levels of the tissues were measured after 2 h (Table 2). The CL level in muscle was too low to measure directly according to the procedure described. Therefore, CL was extracted by homogenizing 20 g muscle with 100 mL 5% acetic acid. The acetic acid solution was separated by centrifuging and the precipitate was further extracted with three 50 mL portions of 99% ethanol. The extracted solutions were pooled and condensed under vacuum at <30°C, and then lyophilized. The residue was used as the assay sample.

Experiments were also designed to measure the recovery of CL from the various samples. One hundred μg of CL was added to 1 g control tissue and its recovery in the standard or modified (muscle) procedure was determined (Table 2). The CL level assayed was then adjusted on the basis of the recovery percentage. As shown in Table 2, CL was found in all tissues tested.

The serum CL levels of rainbow trout kept in drug baths of different CL concentrations were measured; serum level

Table 1. Accuracy and precision of enzyme immunoassay of colistin

Sample	Added, ng/tube	Found,[a] ng/tube	Rec., %	CV,[b]%	N[c]
Within-day assay	3	3 ± 0.50	100.0	16.7	8
	10	10.0 ± 1.10	100.0	11.0	8
	30	30.5 ± 5.43	101.7	17.8	8
	100	102.4 ± 15.09	102.4	14.7	8
Between-day assay	3	2.94 ± 0.71	98.0	24.2	5
	10	10.3 ± 2.15	103.2	20.8	5
	30	31.6 ± 9.62	105.3	30.4	5
	100	100.1 ± 17.15	100.1	17.1	5
Fish serum (50 μL), within-day assay	3	3.08 ± 1.48	102.7	48.1	3
	10	10.3 ± 0.6	103.0	5.8	3
	30	30.0 ± 4.8	100.0	16.0	3
	100	105.7 ± 22.3	105.7	21.1	3

[a]Mean ± SD.
[b]% Coefficient of variation.
[c]Number of assays.

Table 2. Distribution of CL in rainbow trout tissues and fluids 2 h after injection of 1 mg CL into efferent branchial artery (fish weighed about 110 g each)

Tissue	CL detected, μg/g tissue[a]	Rec., [b]%
Muscle	0.37 ± 0.04	40.6
Liver	9.40 ± 1.59	38.5
Kidney	32.16 ± 10.83	27.2
Spleen	12.57 ± 2.15	32.9
Serum[c]	44.60 ± 13.46 (μg/mL)	92.3
Bile[d]	2.24 ± 0.51 (μg/mL)	—

[a]Mean ± SD (n = 5). Results adjusted for recoveries shown, except on bile for which recovery was not performed.
[b]100 μg CL was added to 1 g control tissue and recovery was determined by the procedure described (modified procedure for muscle tissue).
[c]5 μL used for assay.
[d]10 μL used for assay.

variation with time is shown in Figure 5. The level reached the maximum within 2 h.

Serum CL levels were then measured for fish kept for 2 h in baths of 10, 100, and 200 μg CL/mL and were 0.66 ± 0.23, 3.50 ± 0.28, and 5.60 ± 0.95 μg/mL, respectively. These serum CL levels were only 2.8–6.6% of the bath concentration.

Discussion

CL, a peptide antibiotic, is a potent agent for gram-negative microorganisms and is used in veterinary drugs for animals and fish in Japan. As part of a series of studies on the detection of residual drugs in food-related products by sensitive enzyme immunoassay (13, 20), an enzyme immunoassay for CL was developed based on novel methods for preparing a hapten–protein conjugate and an enzyme-labeled hapten using a combination of 2 hetero-bifunctional reagents (11). Both methods are based on thiol addition to the maleimide function, as originally developed by several investigators (21, 22).

The preparative method for CL immunogen was very mild and gave a homogeneous conjugate as judged by SDS electrophoresis. CL shows no distinct ultraviolet absorption, and the exact number of CL molecules coupled per BSA was not determined, although for most hapten–MBS–BSA conjugates prepared by this method the coupling number of hapten molecules is about 15 (19; unpublished data). Both rabbits immunized with the conjugate produced highly specific and sensitive antibodies to CL.

CL was labeled according to the convenient labeling method (18) in a continuous 2-step process using another cross-linker, GMBS. GMBS differs from MBS in the substitution of *N*-butyric acid for benzoic acid, to avoid cross-reaction of CL antibody with MBS, because MBS was used as the cross-linker for preparation of the CL immunogen.

Using anti-CL serum and CL–β-Gal as a tracer, we developed the sensitive double-antibody EIA for quantitation of CL described in this report. The optimal assay procedure was established through testing the reagent concentrations and the incubation periods.

This assay can sensitively and reproducibly detect 3 ng/tube, as shown by an overall mean coefficient of variation of 19.1% for within- and between-day assays at 4 different CL levels. This EIA is specific to CL. Only 3 antibiotics were examined for cross-reactivities by this EIA, as shown in Figure 4, because a minor modification at a functional group in a hapten molecule gave a large reduction in the affinity of anti-hapten antibody (19, 23), and structurally different antigens always showed negligible cross-reactivities.

The immunoassay was used to follow CL levels in several fish organs after the drug had been injected i.a. at a dose of 1 mg/fish. The CL level was highest in kidney tissue and lowest in muscle tissue (Table 2). Serum CL levels of rainbow trout, kept in baths of different CL concentrations, were also measured.

This EIA permits not only easy detection of the residual drug but also easy quantitation of CL in rainbow trout serum as well as tissue such as muscle and liver. A number of samples can be tested at the same time. EIA methods are one of the most promising ways for easily detecting residual drugs in foods and their related products.

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Liquid Chromatographic Monitoring of the Depletion of Carbadox and Its Metabolite Desoxycarbadox in Swine Tissues

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A liquid chromatographic method was used to monitor a depletion study of carbadox (and its most important metabolite, desoxycarbadox) in young pigs fed carbadox-treated rations for 1 week. Carbadox was found in blood (20 ppb), blood serum (26 ppb), and muscle tissue 24 h after withdrawal from treated ration; residues were reduced to a trace (< 2 ppb) in 48 h, and eliminated by 72 h. Desoxycarbadox, although not detected in blood, was found in muscle (17 ppb) 24 h after withdrawal; it was reduced to 9 ppb at 48 h and to a trace by 72 h. Although no carbadox was detected in liver 24 h after withdrawal, appreciable desoxycarbadox (125 ppb) was found in liver 24 h after withdrawal; it was reduced to 17 ppb at 48 h and to a trace by 72 h. Whereas only a trace of carbadox was found in kidney 24 h after withdrawal, 186 ppb desoxycarbadox was found in kidney at 24 h, 34 ppb at 48 h, and a trace at 72 h. No metabolite of carbadox other than desoxycarbadox was found in extracts of swine tissues during this medicated feed trial, and no metabolite was found in blood extracts by using the established methodology. The effect of tissue storage (aging) at −20°C on levels of the drug and its metabolite was a modest alteration of residue levels. The inadvertent use of feed adulterated with furazolidone and initially medicated with chlortetracycline, sulfamethazine, and penicillin G, did not affect the uptake of carbadox in this depletion study or interfere with the analytical methodology.

During the final developmental stages of the recently published liquid chromatographic (LC) analysis for carbadox, desoxycarbadox, and nitrofurazones in pork tissues (1), a preliminary carbadox feeding trial involving 2 piglets (one test, one blank) was undertaken to assess the adequacy of the newly developed LC method for assay of fresh pork tissues for carbadox residues and one of its metabolites, desoxycarbadox. The method was tested for ability to detect residues arising from in vivo incorporation compared with in vitro (spiked) fortification.

We also wished to assess the utility of the method for analyzing blood samples for carbadox and desoxycarbadox residues. Such a procedure might prove valuable to veterinarians and health inspectors in determining that live hogs were free of such drug residues before slaughter.

As a result of the interest generated by the preliminary feeding trial data as well as a need for independently determined data on carbadox body burden for regulatory information, a second, larger carbadox feeding trial, involving 4 piglets and a shortened, 1-week feeding duration, was undertaken to verify preliminary data. In the second trial, both blood and blood serum samples were analyzed to determine what effect blood clotting would have on carbadox and desoxycarbadox levels in whole blood and its serum. The second trial also generated a source of known, fresh tissue samples for assessing the effect of storage (aging) on carbadox and desoxycarbadox levels.

Swine Carbadox Feeding Trial

Medicated ration.—Medicated swine ratio was prepared by the Animal Resources Division, Health Protection Branch, by blending carbadox directly into commercially prepared ration (crumbles) and using a feed mixer to provide ca 50 g/ton (2000 lb) (0.0055%) of finished feed. A portion was retained for assay.

Piglets.—Four piglets (2 males, 2 females), ca 7 weeks old and 10 kg each, were obtained from the same litter, free from any antibiotic exposure. All pigs were fed the regular, carbadox-free ration for a period of adjustment. The usual animal medical assessments were made by the Animal Resources Division as required by their protocol before clearance was given for the feeding trial.

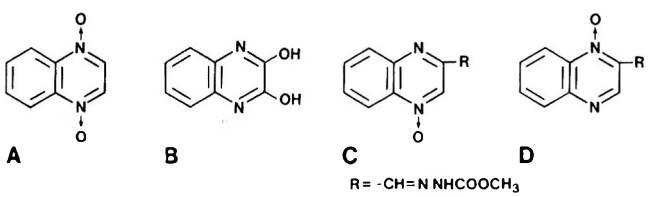
Blood samples.—Blood serum was collected during diagnostic assessment of the piglets, before the feeding trial. Whole blood and blood serum samples were obtained from all pigs (including the control) 24 h after withdrawal from the treated ration. Additional blood samples were collected just before slaughter at 48 or 72 h, respectively, refrigerated until analyzed, and then frozen.

Tissue samples.—Muscle, kidney, and liver tissues, removed from each pig at slaughter, were analyzed by the previously developed LC method (1). Fat was removed from fresh tissues before each was cut into small pieces (less than 1 cu. cm) with kitchen scissors. All material cut from each tissue was pooled, the material was stirred with a spatula to make a uniform sample, then aliquots (10 g) were weighed and individually wrapped in plastic film before being stored at −20°C.

Experimental

The reagents, apparatus, and LC conditions were the same as used in the previous tissue study (1), and the same stringent precautions were observed in cleaning glassware, restricting light, and handling suspected carcinogenic material.

As a result of installing a new, but identical, column [Brownlee RP-10A, reverse phase, C-8(RP-8)] and effecting minor repairs to the LC system prior to undertaking this study, a new set of calibration curves was established as previously reported (1).



LC characterization of other possible carbadox decomposition products.—Four potential decomposition products of carbadox: quinoxaline-N^1, N^4-dioxide (A), dihydroxyquinoxaline (B), methyl-3-(2-quinoxalinylmethylene)carbazate-N^4-oxide (C), and methyl-3-(2-quinoxalinylmethylene)carbazate-N^1-oxide (D) were obtained from R. G. Luchtefeld (Food and Drug Administration, Kansas City, MO 64106, personal communication, 1982) for LC characterization under our conditions.

Blood Analysis

Reagents.—Carbadox and desoxycarbadox (Pfizer Canada, Inc., 17300 Trans-Canada Hwy, Kirkland, Quebec, Can-

[1]Direct inquiries to this author.

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Table 1. LC analysis of preliminary swine feeding trial samples[a]

Tissue	Time of anal.	Carbadox ppb	Carbadox Peak ht, mean	Carbadox SD	Carbadox CV,%	Desoxycarbadox ppb	Desoxycarbadox Peak ht, mean	Desoxycarbadox SD	Desoxycarbadox CV,%
				Control Pig[b]					
Serum	mid.	none	—	—	—	none	—	—	—
Serum	kill	none	—	—	—	none	—	—	—
Muscle	kill	none	—	—	—	none	—	—	—
Liver	kill	none	—	—	—	none	—	—	—
Kidney	kill	none	—	—	—	none	—	—	—
				Treated Pig[b]					
Serum	mid.	16	124	2.9	2.3	none	—	—	—
Serum	kill	19	146	6.6	4.5	none	—	—	—
Muscle	kill	5	44.3	3.9	8.8	34	162.3	32.8	20
Liver	kill	none	—	—	—	50	240	53.5	22
Kidney	kill	<2	—	—	—	94	451	40.7	9
Liver[c]	kill	none	—	—	—	54	258	12.9	5.0

Note: CV = SD × 100/Peak Ht Mean.
[a]Based on antibiotic-free ration treated with carbadox (50 g/ton of finished feed) and zero withdrawal time following 3-week feeding duration.
[b]Both pigs of this trial were females from the same litter.
[c]Repeat assay performed in absence of incandescent light as described earlier (1).

Table 2. Carbadox[a] levels (ppb) in swine blood and blood serum by LC analysis

Gender	Withdrawal, h	Before treatment Serum	Before treatment Blood	24 h Serum	24 h Blood	48 h Serum	48 h Blood	72 h Serum	72 h Blood
Male	24	none	NS[b]	26	19				
				26	20				
Female	48	none	NS	28	22	<2	<2		
				29	24	<2	<2		
Female	72	none	NS	34	31	NS	NS	none	none
				35	32				
Male	control	none	NS	none	none	NS	NS	none	none
				none	none				

[a]No desoxycarbadox was found in any sample.
[b]No sample.

ada, H9R 4V2); 1M KH_2PO_4 solution; ethyl acetate (glass-distilled, LC grade).

Apparatus.—Rotary shaker (Labquake, Labindustries-Canadian Laboratory Supplies); bench-top centrifuge (International, Model HN-S).

Assay procedure.—Pipet 2 mL blood (or serum) into 15 mL screw-cap tube. Add 1 mL 1M KH_2PO_4 solution and 10 mL ethyl acetate. Extract 15 min on rotary shaker; centrifuge 10 min at 2500 rpm in bench-top centrifuge.

Remove ethyl acetate layer to small evaporating flask, and repeat extraction with 10 mL ethyl acetate (15 min), centrifuge (10 min), and combine the ethyl acetate layers. Evaporate extracts to dryness, dissolve residue in 500 μL mobile phase, and inject 100 μL onto LC column.

Tissue Analysis

Prepare, extract, clean up, and analyze muscle, liver, and kidney tissue samples as described previously (1).

Feed Analysis

Reagents.—Dimethylformamide (BDH Analytical Reagent) diluted to 95% with water; aluminum oxide, 80–200 mesh (Baker Analyzed Reagent suitable for chromatographic use; pH of 10% slurry at 25°C is 6.3); methanol (glass-distilled, LC grade).

Apparatus.—Chromatographic column, 1 × 30 cm glass, restricted at one end to ca 4 mm; Büchner funnel, 300 mL capacity.

Assay procedure.—Prepare extract of feed by method of Thorpe (2); then filter mixture through coarse filter paper, and transfer 15 mL filtrate onto chromatographic column containing ca 5 g alumina, prepared as follows: shake alumina (100 g) in water (500 mL) for 30 min, filter mixture in Büchner funnel, and rinse to dry with methanol (3 × 50 mL). Plug column with glass wool, and gently introduce prepared alumina into column with tapping.

Collect eluate, and inject directly 2 μL onto LC column.

Mass Spectrometric Analysis of Furazolidone

Apparatus.—Hewlett-Packard 5985A mass spectrometer.

Sample preparation.—Extract control feed (10 g) by using Thorpe method (2). Subject extract to LC analysis, collect eluate corresponding to furazolidone, evaporate under nitrogen, and subject residue to probe MS analysis.

Microbiological Analysis of Control Feed for Chlortetracycline

Preparation of extract.—(Unpublished method (1983), Lyse Larocque, Bureau of Drug Research, Health Protection Branch, Health and Welfare Canada). Prepare extract of feed by blending 20 g feed in 80 mL acidified acetone (4N HCl–acetone–water: 1 + 13 + 6). Stir mixture 2 min, let settle 2 min, and stir again. Adjust pH to 1.0–1.2 with 1N HCl (4 drops). Blend mixture 3 min at high speed, then centrifuge 15 min at 2000 rpm. Adjust pH of supernate to 4.0 with 1N NaOH solution before proceeding with microbiological assay or Bratton-Marshall test.

Table 3. LC monitoring of carbadox depletion and metabolism to desoxycarbadox in swine[a]

	Carbadox, ppb				Desoxycarbadox, ppb			
Tissue	Control	24 h	48 h	72 h	Control	24 h	48 h	72 h
Serum	none	26	<2	none	none	none	none	none
Blood	none	20	<2	none	none	none	none	none
Muscle	none	19	<2	none	none	17	9	<2
Liver	none	none	none	none	none	125	17	<2
Kidney	none	2	<2	none	none	186	34	<2

[a]Assay levels below 10 ppb sensitivity are unreliable; precision ± 5 ppb.

Table 4. Effect of storage on residue levels of carbadox and desoxycarbadox, analysis by LC

		Carbadox, ppb					Desoxycarbadox, ppb				
Pig	Tissue	Kill	1 week	2 weeks	3 weeks	4 weeks	Kill	1 week	2 weeks	3 weeks	4 weeks
	Blood	none					none				
	Serum	none					none				
Control	Muscle	none	none	none	none	none	none	none	none	none	none
	Liver	none	none	none	none	none	none	none	none	none	none
	Kidney	none	none	none	none	none	none	none	none	none	none
	Blood	26					none				
	Serum	20					none				
24 h	Muscle	19	14	9	12	4	17	15	15	28	16
	Liver	none	none	none	none	none	125	133	133	115	72
	Kidney	2	<2	<2	<2	none	186	91	112	130	92
	Blood	<2					none				
	Serum	<2					none				
48 h	Muscle	<2	<2	none	none	<2	9	4	6	6	5
	Liver	none	<2	none	none	none	17	16	4	10	12
	Kidney	<2	—[a]	<2	none	none	34	10[a]	28	29	25
	Blood	none					none				
	Serum	none					none				
72 h	Muscle	none	<2	none	none	none	<2	<2	none	none	none
	Liver	none	none	<2	none	none	<2	3	<2	2	2
	Kidney	none	<2	<2	none	none	<2	<2	<2	<2	none

[a]Problems with extraction; unable to measure accurately.

Analysis.—Use standard, diffusion, agar-plate microbiological method (3).

Modified Bratton-Marshall Test for Sulfamethazine

The Bratton-Marshall test (4) for sulfa drugs was modified as follows for testing presence of sulfamethazine in control feed extract:

Treat feed extract (3 mL), as prepared above, with 0.1% sodium nitrite solution (1.0 mL) and concentrated HCl (1.0 mL) and let stand 3 min. Treat mixture with 0.2% ammonium sulfamate solution (1.0 mL) and let stand an additional 3 min. Shake, and treat with 1.0 mL 0.1% *N*-(1-naphthyl)ethylenediamine dihydrochloride (NED, dye reagent) solution; rosy-pink color indicates positive result.

Results and Discussion

Swine Carbadox Feeding Trials

Preliminary trial.—In the preliminary study, one piglet was maintained on carbadox-treated ration (50 g/ton finished feed) for 3 weeks before slaughter; the other piglet, used as the control for blank samples, was fed the same ration without carbadox. At slaughter (zero withdrawal time), blood serum of the test pig contained 19 ppb carbadox but no desoxycarbadox (Table 1); muscle tissue contained 5 ppb carbadox and 34 ppb desoxycarbadox; the liver contained no carbadox but contained 50 ppb desoxycarbadox; and the kidney, surprisingly, showed only a trace of carbadox (<2 ppb) but 94 ppb desoxycarbadox, practically double that of liver, the generally recognized target organ (5).

Blood and tissue analysis for major trial.—Table 2 summarizes the carbadox levels found in the blood and blood serum of each of the 3 test pigs and the control pig 24 h after withdrawal of carbadox-treated ration. Table 2 also indicates the gender of each pig together with the corresponding withdrawal time (or control), and the level of carbadox in blood and blood serum of each pig at the time of slaughter.

The results of the depletion study for carbadox and desoxycarbadox levels in blood and tissues of pigs fed carbadox-medicated ration are summarized in Table 3 for the effect of withdrawal time on residue levels, and in Table 4 for the effect of storage time on residue levels. Data presented in these and other tables discussed herein are based on the calibration curve determinations of Table 5.

The carbadox level in blood and in blood serum 24 h after withdrawal of the medicated feed (Table 3) is essentially the same, within the allowed precision of analysis (± 5 ppb). The blood carbadox level found in this study after 24 h withdrawal is also essentially identical to that found in blood serum (19 ppb) at zero withdrawal time in the preliminary study (Table 1) conducted with the same level of carbadox medication in the ration consumed over 3 weeks. These carbadox blood data serve to confirm the belief that pigs ingesting 50 g carbadox/ton finished feed would achieve a steady-state blood carbadox level in 1 week. The separate blood and serum analyses (Tables 2 and 3) also confirm the preliminary finding of no desoxycarbadox in blood serum, and provide evidence that neither carbadox nor desoxycarbadox was lost from blood through clotting to obtain serum. The blood data also show that carbadox is quickly reduced to a trace at 48 h and eliminated from the blood by 72 h.

The analysis of muscle shows comparable levels of carbadox (19 ppb) and desoxycarbadox (17 ppb) at 24 h withdrawal time (Table 3) compared with the previous levels of 5

Table 5. Calibration curves for reference compounds[a]

Amt, ng	Carbadox Peak ht, mm	Carbadox Std dev.	Carbadox CV, %	Desoxycarbadox Peak ht, mm	Desoxycarbadox Std dev.	Desoxycarbadox CV, %	Nitrofurazone Peak ht, mm	Nitrofurazone Std dev.	Nitrofurazone CV, %	Furazolidone Peak ht, mm	Furazolidone Std dev.	Furazolidone CV, %
2	26.1	1.1	4.0	17.1	0.9	5.3	31.4	0.8	2.5	26.9	1.0	3.8
10	115	3.5	3.1	76	4.0	5.3	139	2.4	1.8	121	2.6	2.1
20	223	6.9	3.1	150	8.1	5.4	271	3.8	1.4	238	4.6	1.9
30	305	11.9	3.9	216	11.5	5.3	376	9.7	2.6	333	8.2	2.5
40	384	14.3	3.7	276	13.3	4.8	475	13.6	2.9	428	12.4	2.9
	$y = 9.4x + 18.6$ corr. = 0.997			$y = 6.8x + 7.4$ corr. = 0.999			$y = 11.7x + 20.1$ corr. = 0.998			$y = 10.5x + 14.2$ corr. = 0.999		

[a]All peak heights normalized for attenuation setting 4; average of at least 2 injections using 2 dilutions of 3 solutions.

and 34 ppb, respectively, of the preliminary study obtained at zero withdrawal time. It is not clear why the muscle level of carbadox at 24 h should be higher than at zero withdrawal time unless the difference is due to gender; however, the muscle carbadox burden is comparable to that of the blood for the male pig. The lower desoxycarbadox level (one-half) in muscle at 24 h compared with 34 ppb of the preliminary study at zero time could be accounted for by the rate of elimination (50% every 24 h) of desoxycarbadox as seen for muscle from 24 to 74 h (Table 3). Carbadox appears to be eliminated from muscle even more quickly.

The apparent absence of carbadox burden in liver (Table 3) supports the similar, preliminary finding; however, the much higher level of desoxycarbadox (125 ppb) in liver at 24 h compared with the preliminary, zero time (50 ppb) (Table 1) is difficult to account for, especially when no desoxycarbadox is found in the blood and when the zero time level (50 ppb) was obtained after feeding for 3 weeks instead of 1 week. Desoxycarbadox, however, appears to be rapidly eliminated from the liver (Table 3). Satisfactory understanding of such variation would require additional studies involving a larger number of animals.

Only a trace of carbadox was found in the kidney (Table 3), confirming the preliminary finding. The kidney, however, with 186 ppb desoxycarbadox at 24 h (Table 3 and Figure 1)—twice the 94 ppb level found at the preliminary, zero time—must be recognized as the target organ, as found in the preliminary trial, in place of liver, the generally regarded target organ (5).

Effect of Storage Time on Tissue Residue Levels

The results of analyzing uniform aliquots of muscle, liver, and kidney for carbadox and desoxycarbadox at weekly intervals over a period of one month are summarized in Table 4.

Storage time appears to result in a modest decrease in carbadox content in muscle which might be attributed to the effect of more tenacious drug retention by the tissue as it ages, dehydrates, and undergoes slow, physical alteration during storage at −20°C. Because of the virtual absence of carbadox in both liver and kidney at slaughter (24 h withdrawal), no significant trends were noted for carbadox residues in these organs over time.

Desoxycarbadox levels in muscle and liver tissue taken 24 h after the withdrawal time appear to show a modest elevation on storage followed by a modest decrease. A similar phenomenon appears to hold also for kidney following an initial decrease of desoxycarbadox in the first week of storage. Alteration of the tissues through physical change, dehydration, etc., during storage at −20°C may result in the release of originally bound and unavailable desoxycarbadox, i.e., the reverse effect noted for carbadox. (Carbadox, being more polar than desoxycarbadox, would likely be more readily bound by tissue.) It is unlikely that enzymatic release of drug could account for such changes either during storage at −20°C or during the short (5 min) thawing and sample workup period.

No significant changes were noted for desoxycarbadox levels on storage of tissue taken 48 h after slaughter presumably because the longer withdrawal time had already reduced the metabolite concentration to a relatively low level.

Effect of Long-Term Storage on Blood Serum and Tissues

Muscle, liver, and kidney tissues and blood serum analyzed for carbadox and desoxycarbadox in the preliminary feeding trial were stored at −20°C for 7 months and re-analyzed as part of this study to assess the effect of longer term storage. The results of Table 6 show an increase of carbadox level (+ 12 ppb) in serum perhaps caused by sample concentration through dehydration during storage. Since the carbadox lev-

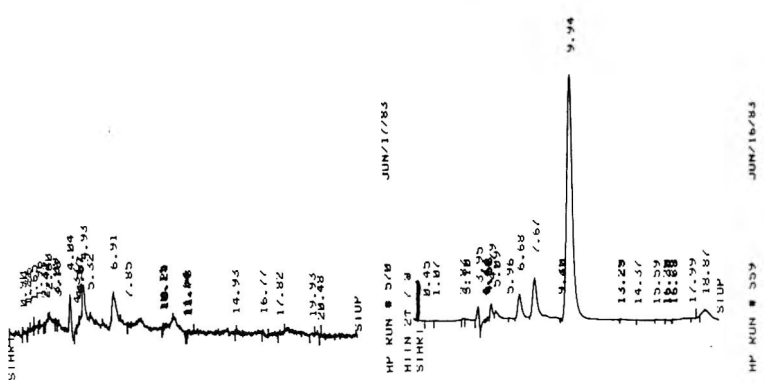

Figure 1. LC tracings for pork kidney tissues: control sample (left) showing minimal background at attenuation setting 4, and test sample (right), obtained 24 h after withdrawal of carbadox-treated ration and recorded at attenuation setting 7, showing trace of carbadox (R_t 5.09 min) and 186 ppb desoxycarbadox (R_t 9.94 min). Each sample injection was 100 μL.

Table 6. Effect of prolonged storage on residue levels in blood serum and tissue samples

Sample	Carbadox, ppb			Desoxycarbadox, ppb		
	Sept/82	Apr/83	Diff.	Sept/82	Apr/83	Diff.
Control:						
Serum	nil	nil	none	nil	nil	none
Muscle	nil	nil	none	nil	nil	none
Liver	nil	nil	none	nil	nil	none
Kidney	nil	nil	none	nil	nil	none
Treated:						
Serum	19	31	+12	nil	nil	none
Muscle	5	trace	−5	34	38	+4
Liver	nil	trace	+	50	60	+10
Kidney	trace	nil	—	94	59	−35

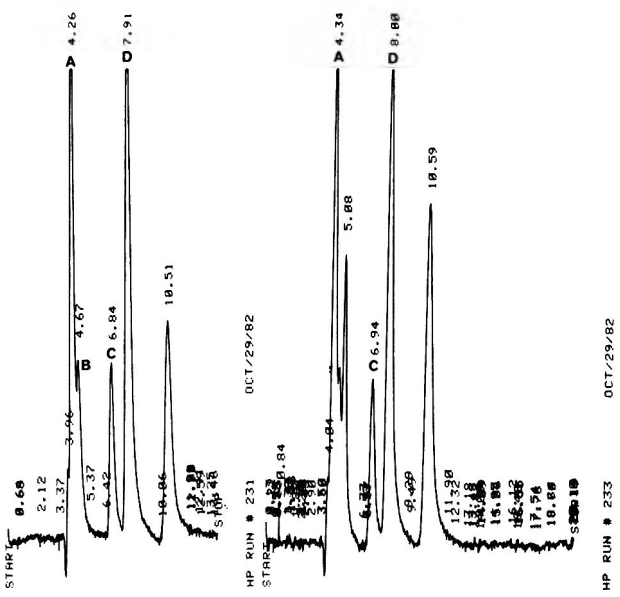

Figure 2. LC tracings of composite solution of possible carbadox metabolic products including desoxycarbadox (left), and re-injection of same composite followed by co-injection of 10 ppb carbadox (R_t 5.08 min) and 10 ppb desoxycarbadox (R_t 10.59 min) (right). Each injection was 20 μL, and attenuation setting 4 was used for each recording. (See text for identity of A, B, C, and D.)

els in muscle, liver, and kidney tissues were originally low (trace levels, i.e., below the stated level of reliable measurement), no significant change of carbadox level was observed in these samples. No significant change of desoxycarbadox levels was seen with muscle tissue, but a modest increase was observed in liver likely due to dehydration of the liver during storage. A significant decrease in desoxycarbadox level was observed with kidney tissue after 7 months of storage. Perhaps degeneration of the finely chopped tissue and denaturization of protein results in binding of some of the original desoxycarbadox, or perhaps the metabolite is degraded by enzymes and/or other chemicals present in kidney.

LC Characterization of Other Possible Carbadox Decomposition Products

The results of LC characterization of the 4 possible decomposition products of carbadox (quinoxaline-N^1,N^4-dioxide (A), dihydroxyquinoxaline (B), methyl-3-(2-quinoxalinylmethylene)carbazate-N^4-oxide (C), and methyl-3-(2-quinoxalinylmethylene)carbazate-N^1-oxide (D)) under identical conditions used for tissue analysis, are illustrated in Figure 2. Solutions of the decomposition products were prepared of the approximate concentration 50 μg/50 μL mobile phase (acetonitrile–0.01M ammonium acetate–ethanol 25 + 70 + 5). A similar elution order was found for the 4 compounds as reported by Luchtefeld (6) under somewhat different LC conditions and correspondingly different retention times.

No peaks were found in any LC chromatograms of the tissue or blood extracts that could be attributed to any of the additional, possible carbadox decomposition products.

Feed Analysis

The results of LC analysis of in-house prepared carbadox-treated swine ration and of the corresponding control ration, following extraction of samples of each by the method of Thorpe (2) are shown in Figure 3. Analysis of the treated ration confirmed the desired level (0.0046%) of carbadox medication but revealed the presence of another substance identified by retention time as furazolidone (0.0009%). LC analysis of the control ration also revealed the same level of furazolidone whose identity was confirmed by in-house mass spectrometric analysis (Figure 4) of residue obtained by collection of the LC eluant.

Independent analysis of the treated and control rations by the Feed and Fertilizer Laboratory, Agriculture Canada, for carbadox by a colorimetric procedure confirmed the carbadox level (0.0049%) and verified the presence of furazolidone by LC analysis (no quantitation).

Investigation of Feed Labeling

Since a feed label, supplied at the same time of the second request for additional feed samples for analysis, indicated that the feed supply was medicated with chlortetracycline

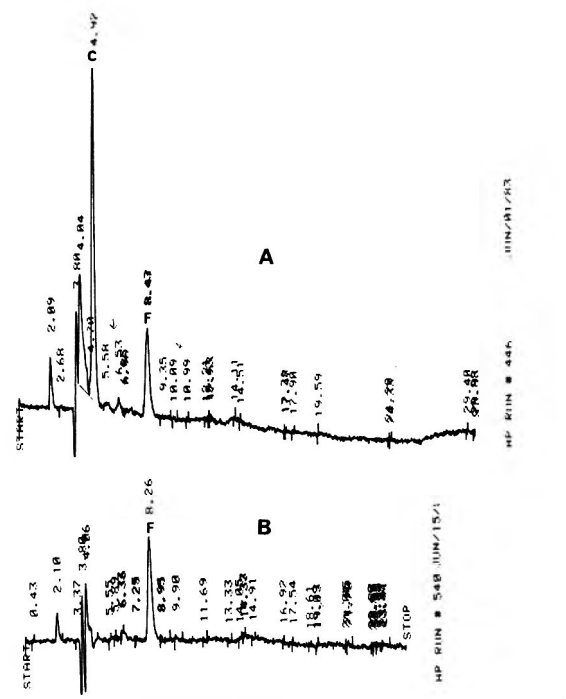

Figure 3. LC analysis of treated ration (A) and control ration (B), each extracted with 95% DMF (2), showing carbadox (C) and furazolidone (F) in A and furazolidone (F) only in B.

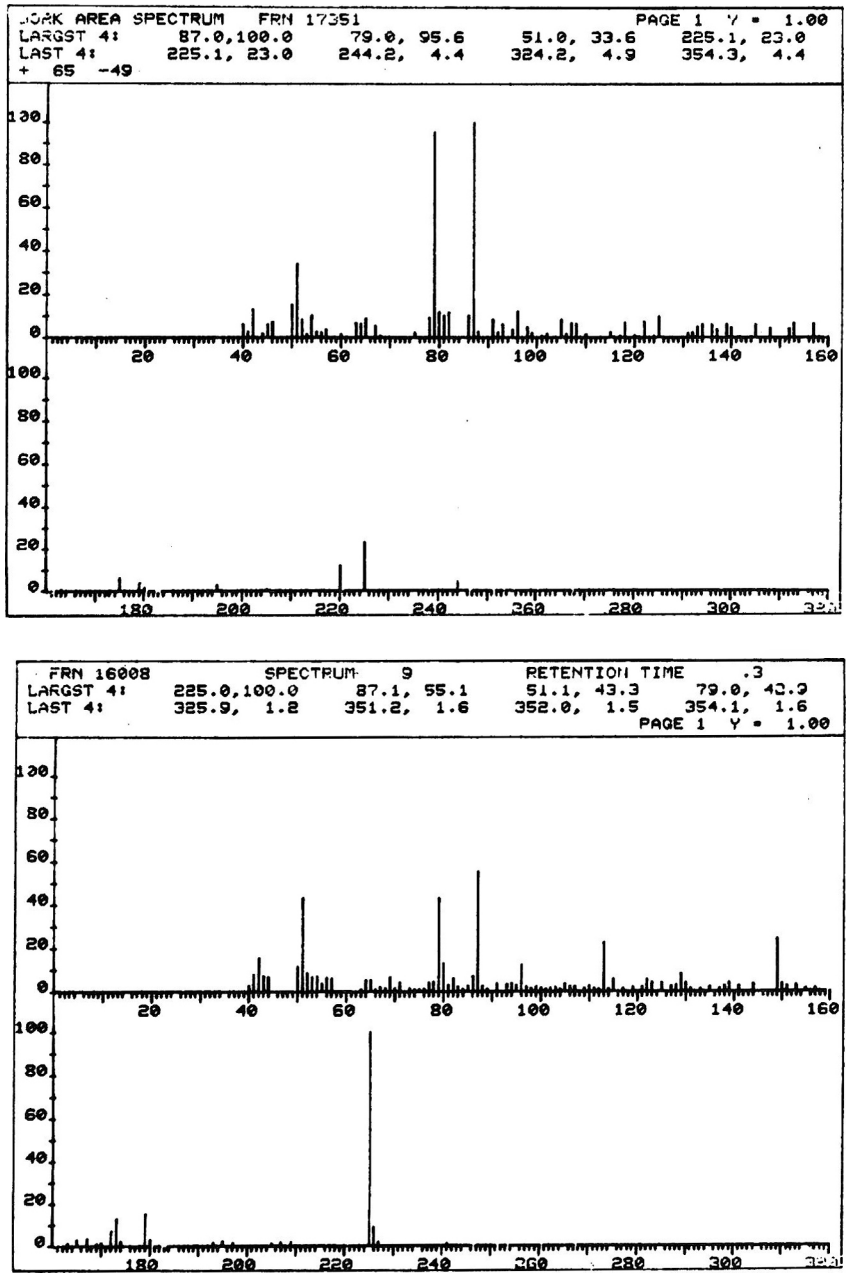

Figure 4. EI mass spectra (70 eV) of suspected furazolidone residue collected from LC analysis (top) and furazolidone standard (bottom).

(0.011%), sulfamethazine (0.011%), and penicillin G (0.0055%), a second LC analysis was performed on the extract prepared for microbiological testing. Standard solutions of the above 3 antibiotics were prepared in the mobile phase and subjected to LC analysis under the conditions of this study. While sulfamethazine gave a good response at 350 nm, no response was obtained from the other 2 drugs. Figure 5 shows the results of LC analysis of sulfamethazine standard, sulfamethazine and furazolidone in the control feed, and the confirmation effect of spiking the control feed sample with sulfamethazine. The presence of sulfamethazine in the feed supply was also supported by the result of a positive Bratton-Marshall test (4) performed on an extract of the feed.

The presence of chlortetracycline in the feed supply was confirmed by means of specific microbiological analysis for chlortetracycline (3) performed on the same feed extract.

Work schedules precluded analyzing for penicillin G; however, the feed extract has been retained frozen for possible later investigation.

Non-detection of chlortetracycline and penicillin G under the LC conditions of this study may be accounted for by the fact that penicillin G is weakly UV-absorbing at 350 nm, and chlortetracycline, with maximum absorption at 380 nm and expected appreciable absorption at 350 nm, apparently did not extract from the feed in sufficient quantity for detection.

The presence of the 3 antibiotics chlortetracycline, sulfamethazine, and penicillin G together with the adulterant furazolidone in the feed supply, although not appearing to have interfered with the uptake of carbadox by the pigs, may have exerted a synergistic effect resulting in the observed much greater burdening of liver and kidney tissue with the metabolite, desoxycarbadox, at 24 h withdrawal time than found at zero withdrawal time, all other factors being equal. From an analytical concern, it should be noted that the presence of chlortetracycline, sulfamethazine, penicillin G, or furazolidone did not interfere with our LC methodology (1), developed as a screening procedure for carbadox, desoxycarbadox, furazolidone, and nitrofurazone.

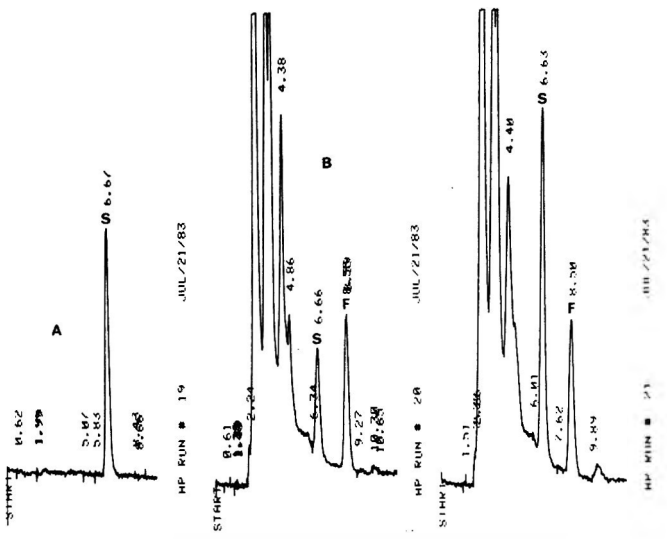

Figure 5. LC analysis of sulfamethazine standard (A) (10 μL of 0.011% solution), extract of control feed (B) (2 μL of prepared extract) showing presence of sulfamethazine (S) and furazolidone (F), and sample of same feed extract (2 μL) spiked with sulfamethazine standard (S) (10 μL, 0.011%). Earlier peaks arise from other soluble feed material.

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FRUIT AND FRUIT PRODUCTS

Interlaboratory Variability of Methods Used for Detection of Economic Adulteration in Apple Juice

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Thirteen laboratories participated in a study to determine the interlaboratory variability of 4 methods used to detect economic adulteration in apple juice. The methods included the determination of individual sugars, 5-(hydroxymethyl)-2-furaldehyde, chlorogenic acid, organic acids, and L-malic acid. The coefficients of variation found depended on the method and level of analyte, but were often 5% or less. At this point, the most important test for detecting economic adulteration is the total malic/L-malic acid ratio. Any ratio of 0.9 or less would indicate a nonauthentic sample. The 0.9 ratio represents an addition of 20% synthetic malic acid in the unknown juice sample. A 0.75 ratio would indicate 50% adulteration, while a 0.5 ratio would indicate a totally synthetic juice. Fumaric acid, a minor contaminant in synthetically produced malic acid, shows great promise as an indicator of economic adulteration. The results support the view that the methods are now ready for AOAC collaborative study.

A number of methods have recently been published for detecting economic adulteration in apple juice (1–3). These include analysis of sugars, sugar ratios, carbon isotope ratios, polyphenols, organic acids, sorbitol, and proline. Together, these tests give useful information about the quality and authenticity of a particular apple juice sample. An individual laboratory can perform these assays very precisely. But what is the expected variability when more than one laboratory performs these analyses on the same sample? Interlaboratory variability can be important when a regulatory agency and a commercial firm report apparently conflicting results or results that indicate that a juice is on the borderline between authentic and adulterated.

The purpose of this study is to determine the interlaboratory variability of certain analytical methods that are commonly used to detect adulteration in apple juice.

Protocol for the Interlaboratory Study

Four samples were supplied to the participating laboratories: 3 different types of juice and a blind duplicate. Each sample consisted of 4 plastic vials containing about 15 mL juice. Four bottles of each sample were supplied to minimize the possibility of microbial or mycological contamination of the sample between analyses. The authentic apple juice was designated sample C, the synthetic juice was labeled sample B, the third juice was a mixture of the authentic and synthetic juices; blind duplicates of this juice mixture were designated as samples A and D.

The participants were requested to analyze and quantitate the following compounds: fructose, glucose, sucrose, 5-(hydroxymethyl)-2-furaldehyde (HMF), chlorogenic acid, total malic acid, L-malic acid, and fumaric acid. The analytical methods used for determining the sugars, HMF, and chlorogenic acid were those currently employed in the participating laboratories. The analytical methods used to determine L-malic acid and other organic acids are described in this paper.

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Experimental

Preparation of Apple Juice Samples

The samples consisted of a known authentic juice, a synthetic juice prepared from concentrate, and a mixture of these 2 juices. The authentic juice was apple juice supplied by a member of NFPA (National Food Processors Association). An appropriate amount of 37% formaldehyde was added to this juice so the final concentration was 0.4%. This level is necessary to prevent bacterial or mycological growth.

Table 1. Reported levels of fructose (%) in prepared juice samples

	Apple juice sample			
Lab.	A	B	C	D
6	5.36	4.83	6.10	5.25
11	5.25	4.67	5.64	5.28
12	5.34	4.8	5.88	5.2
15	5.2	4.7	6.0	5.4
17	5.41	4.69	5.69	5.16
19	5.24	4.56	6.86	5.21
20	4.80[a]	4.30[a]	5.40[a]	4.90[a]
22	5.38	4.57	5.89	5.31
27	5.12	4.59	5.64	5.13
32	5.02	4.6	5.65	5.2
33	5.2	4.6	5.7	5.9[b]
35	5.3	5.01	6.0	5.11
38	6.03[a]	5.32[a]	6.59[a]	6.01[a]
Mean	5.256	4.693	5.823	5.225
SD	0.118	0.139	0.167	0.088
CV,[b] %	2.2	3.0	2.9	1.7
SD[c] = 0.102				
CV,[d] % = 2.0				

[a]Not used in statistical summary; eliminated by Thompkins and Willke test (4).
[b]Coefficient of variation.
[c]Intralaboratory SD (estimate).
[d]Intralaboratory CV (estimate).

Table 2. Reported levels of glucose (%) in prepared juice samples

	Apple juice sample			
Lab.	A	B	C	D
6	3.96	5.11	2.82	3.91
11	3.97	5.03	2.84	4.0
12	4.08	4.99	3.1	4.11
15	4.1	5.3	3.1	4.1
17	4.29	5.13	3.07	4.13
19	4.02	5.01	2.90	4.07
20	3.8	4.7	2.90	3.80
22	4.16	4.92	3.03	4.09
27	3.97	4.97	2.85	4.03
32	3.28[e]	5.12	2.0[e]	3.28[e]
33	4.1	5.2	2.8	4.5[e]
35	4.14	4.86	3.17	4.24
38	4.05	4.96	3.02	4.09
Mean	4.053	5.023	2.967	4.052
SD	0.124	0.154	0.129	0.117
CV,[b] %	3.0	3.1	4.3	2.9
SD[c] = 0.049				
CV,[d] % = 1.2				

[a–d]See previous table.
[e]Not used in statistical summary; eliminated by Dixon text.

Table 3. Reported levels of sucrose (%) in prepared juice samples

Lab.	Apple juice sample A	B	C	D
6	1.01	0.50	1.49	1.00
11	1.00	0.50	1.48	1.04
12	1.04	0.57	1.44	1.04
15	1.2	0.7	1.5	1.3
17	1.03	0.50	1.42	0.99
19	1.09	0.56	1.58	1.09
20	1.00	0.30	1.50	0.80
22	1.02	0.49	1.53	1.04
27	0.97	0.49	1.42	0.97
32	1.22	0.71	1.54	1.22
33	1.0	0.5	1.6	1.0
35	1.05	0.42	1.6	0.72
38	1.11	0.58	1.51	1.07
Mean	1.057	0.525	1.508	1.022
SD	0.078	0.107	0.061	0.150
CV,[b] %	7.4	20.4	4.1	14.7
SD[c] = 0.079				
CV,[d] % = 7.6				

[a–d]See previous tables.

Table 4. Calculated glucose/fructose ratios in prepared juice samples

Lab.	Apple juice samples A	B	C	D
6	0.739	1.058	0.462	0.745
11	0.756	1.077	0.504	0.758
12	0.764	1.040	0.527	0.790
15	0.788	1.128	0.517	0.759
17	0.793	1.094	0.540	0.800
19	0.767	1.099	0.495	0.781
22	0.773	1.077	0.514	0.770
27	0.775	1.083	0.505	0.786
32	—[f]	1.113	—[f]	—[f]
33	0.788	1.130	0.491	—[f]
35	0.781	0.970	0.528	0.830
Mean	0.772	1.079	0.508	0.780
SD	0.017	0.045	0.022	0.026
CV,[b] %	2.1	4.2	4.4	3.3
SD[c] = 0.016				
CV,[d] % = 2.0				

[a–e]See previous tables.
[f]Not calculated due to outlier value.

The synthetic juice sample was prepared from an apple juice concentrate that had the characteristics of a totally synthetic product. One part (by weight) concentrate was mixed with 6 parts distilled, deionized water. Again, formaldehyde was added to make a final concentration of 0.4%.

The mixed juice was prepared by mixing equal volumes of the above 2 samples.

The samples were individually packaged and shipped in scintillation vials, with about 15 mL juice in each vial. The vials had previously been washed with detergent, rinsed thoroughly with distilled water, and dried. The samples were frozen before shipment and the participating laboratory was instructed to freeze the samples upon receipt.

Analytical Procedures

The analytical methods used in this study are described below. For organic acids, the procedure was followed exactly; for other compounds, the procedures used were similar but may not have been exactly as described below.

Sugars.—Fructose, glucose, and sucrose were individually quantitated by liquid chromatography (LC) on a Waters carbohydrate analytical column (30 cm × 4.9 mm) under the following conditions: ambient temperature, mobile phase of acetonitrile–water (80 + 20) at 1.5 mL/min, and refractive index detector. Samples were analyzed by diluting with an equal volume of water, filtering through a 0.45 μm filter, injecting 15 μL, and measuring peak heights.

HMF and chlorogenic acid.—These 2 compounds were determined by using an LC method modified from Brause and Raterman (1): reverse phase C-18 column (25 or 30 cm long), ambient temperature, mobile phase of 0.033M KH_2PO_4–methanol–acetic acid (80 + 20 + 4) at 1.0 mL/min, and variable wavelength UV detector at 300 nm. Samples were analyzed by filtering the juice through a 0.45 μm filter and injecting 15 μL.

L-*Malic acid.*—This acid was determined using an enzymatic assay procedure. A commercial kit is available from Boehringer Mannheim Biochemicals (Kit No. 139 064). To minimize pipetting errors, the samples were analyzed in duplicate and the results were averaged.

Organic acids.—The participants were requested to use the following LC procedure (*Anal. Abstr.* **37**, 4F48 (1979)) modified by the U.S. Food and Drug Administration in Buffalo, NY. Malic acid (total) and fumaric acid were determined using a C-18 reverse phase column (25 or 30 cm long) packed with 5 μm spherical particles, under the following conditions: ambient temperature, mobile phase of 0.01M KH_2PO_4 in 0.75% phosphoric acid at 0.8 mL/min, and a UV detector at 214 (or 210) nm. Samples were analyzed by diluting 5.00 g juice with 5.00 g water, mixing thoroughly, filtering through 0.45 μm filter, and injecting 20 μL (or standard loop).

Statistical Treatment of Results

The data for the various analyses are the values reported by the laboratories participating in this study; the ratio values

Table 5. Reported levels of HMF (ppm) in prepared juice samples

Lab.	Apple juice sample A	B	C	D
6	15.0	28.7	1.1	15.7
11	13.9	25.9	1.45	14.0
12	17.0	30.7	7.8[e]	16.0
15	16.0	29.3	1.82	16.1
17	15.5	27.5	0	15.9
19	15	27	0	15
20	15.0	27.0	1.50	15.1
22	14.5	27.5	1.1	14.9
33	11.8	23.0	0.8	10.7[e]
35	17.6	26.3	1	30.3[e]
Mean	15.13	27.29	0.92	15.34
SD	1.62	2.09	0.65	0.72
CV,[b] %	10.7	7.7	70.7	4.7
SD[c] = 0.34				
CV,[d] = 2.2				

[a–e]See previous tables.

Table 6. Reported levels of chlorogenic acid (ppm) in prepared juice samples

Lab.	Apple juice sample A	B	C	D
6	10.0	0	19.3	10.0
11	9.4	0	20.7	9.5
15	11.14[e]	1	23.08[e]	11.42
17	9.4	0	18.5	9.5
19	10	0	20	10
22	10.1	0	20.3	10.1
33	10.1	0	19.5	8.9
35	9.9	0	18.8	8.8
Mean	9.84	—[g]	19.59	9.78
SD	0.31	—[g]	0.81	0.83
CV,[b] %	3.2	—[g]	4.1	8.4
SD[c] = 0.44				
CV,[d] % = 4.5				

[a–f]See previous tables.
[g]Statistics not calcu ated.

Table 7. Calculated HMF/chlorogenic acid ratios in prepared juice samples

Lab.	Apple juice sample A	B	C	D
6	1.50	—[h]	0.057	1.57
11	1.48	—[h]	0.070	1.47
15	—[f]	—[h]	—[f]	1.41
17	1.65	—[h]	0.0	1.67
19	1.50	—[h]	0.0	1.50
22	1.44	—[h]	0.054	1.48
33	1.17	—[h]	0.041	—[f]
35	1.78	—[h]	0.053	—[f]
Mean	1.503		0.0354	1.517
SD	0.189		0.0278	0.091
CV,[b] %	12.6		78.5	6.0
SD[c] = 0.027				
CV,[d] % = 1.8				

[a–g]See previous tables.
[h]Large number; not calculated because of zero in denominator.

Table 8. Reported levels of total malic acid (mg/100 g) in prepared juice samples

Lab.	Apple juice sample A	B	C	D
11	426	529	354	442
12	494[e]	515	544[e]	491[e]
15	405	505	377	417
17	399	527	285[e]	397
19	426	506	344	428
22	442	542	354	445
27	430	507	368	433
32	430[i]	499[i]	379[i]	446[i]
33	391	512	337	410
35	466[a]	613[a]	410[a]	491[a]
Mean	418.6	515.8	359.0	427.3
SD	17.9	14.0	16.1	17.9
CV,[b] %	4.3	2.7	4.5	4.2
SD[c] = 8.1				
CV,[d] % = 1.9				

[a–h]See previous tables.
[i]Value obtained using Bio-Rad HPX-87H column.

(viz, glucose/fructose ratio, HMF/CGA ratio, and L-malic acid/total malic acid ratio) were calculated from the supplied data. All data submitted were first subjected to the Thompson and Willke test and Dixon's test to eliminate outliers (4) before being included in the summary statistics. However, data in the ratio tables were not so treated, and all values therein were included in the statistical calculations. The mean, standard deviation, and coefficient of variation of the data in each table were calculated; these statistics represent a measure of interlaboratory variability of the assay methods.

An estimate of the intralaboratory variability was made from the values for the blind duplicate.[1]

Results and Discussion

Sugars.—The results for determination of individual sugars are very good as can be seen from the data in Tables 1–3. Interlaboratory coefficients of variation (CV) of results for fructose and glucose are 4.3% or less, while those for sucrose range up to 20.4%. The standard deviations for sucrose are similar to those for the other 2 sugars, so the large CV values are probably due to the lower sucrose content in the juice samples.

The coefficients of variation for the glucose/fructose ratios (Table 4) are 4.4% or less, which makes this ratio precise enough to be useful in detecting addition of a sugar solution to authentic apple juice. Data published in the literature suggest an expected range of glucose/fructose ratio in apples and apple juices from 0.16 to 0.67 (1, 5, 6). Summary statistics in Table 4 show that samples A (and its duplicate D) and B have values for the glucose/fructose ratio greater than normal so that these samples would be considered economically adulterated. As prepared, sample A is 50% adulterated and B is entirely nonauthentic. Thus, samples with a high percentage of adulteration can be detected using this ratio. However, because of the wide natural variability for this ratio, the sugar content of a nonauthentic apple juice sample can be adjusted rather easily with non-apple-derived sugars (e.g., high fructose corn syrup, invert beet sugar), so the glucose/fructose ratio is limited in its usefulness for detecting economic adulteration of apple juice.

HMF/chlorogenic acid.—The variability of results for analyses for 5-(hydroxymethyl)-2-furaldehyde (HMF) was greater than that for the sugars (Table 5). The statistical summary shows coefficients of variation of about 10%, except for sample C which has a very low HMF content and a correspondingly higher CV value. Much of the interlaboratory variability may result from the difficulty of quantitating the HMF peak from the chromatogram because, with the mobile phase and column conditions used for this determination, the HMF peak elutes near the solvent front, along with other, interfering peaks. An important observation is that authentic apple juice contains low levels of HMF whereas synthetic samples can contain significantly greater amounts (unpublished data); for example, compare the 0.9 ppm level found in the authentic juice (sample C) with the 27 ppm found in synthetic juice (sample B).

The data obtained for chlorogenic acid (CGA) (Table 6) show an interlaboratory variability of 8.4% or less. Chlorogenic acid is a naturally occurring polyphenolic compound in apples, and the demonstrated lack of this substance in a sample would indicate that the particular juice was not derived from apples. The authentic juice sample used for this study (sample C) contained a normal level of CGA, whereas the synthetic juice (sample B) contained only a trace.

The calculated ratios for HMF/CGA from the individual data sets are shown in Table 7. Authentic samples of apple juice have HMF/CGA ratios of 0.2 or less, whereas nonauthentic samples can have much larger values for this ratio (unpublished data). The statistical summary of these data

Table 9. Reported levels of L-malic acid (mg/100 g) in prepared juice samples

Lab.	Apple juice sample A	B	C	D
6	279	245	311	269
11	294	171[e]	329	297
15	376[a]	336[a]	425[a]	379[a]
17	294	261	323	292
19	291	269	345	314
22	294	250	331	299
32	259	227	293	256
33	286	243	328	280
35	269	280	261	241
38	269	258	307	276
Mean	281.7	254.1	314.2	280.4
SD	13.2	16.5	25.1	22.9
CV,[b] %	4.7	6.5	8.0	8.2
SD[c] = 9.3				
CV,[d] % = 3.3				

[a–e]See previous tables.

[1]The estimate is based on the use of Formula 3 on page 18 of Youden and Steiner (4).

Table 10. Calculated L-malic acid/total malic acid ratios in prepared juice samples

	Apple juice sample			
Lab.	A	B	C	D
11	0.69	—[f]	0.93	0.67
17	0.74	0.50	—[f]	0.74
19	0.68	0.53	1.00	0.73
22	0.67	0.46	0.94	0.67
32	0.60	0.45	0.77[f]	0.57
33	0.73	0.47	0.97	0.68
Mean	0.685	0.482	0.960	0.677
SD	0.050	0.033	0.032	0.061
CV,[b] %	7.3	6.8	3.4	8.9
SD[c] = 0.023				
CV,[d] % = 3.4				

[a–f]See previous tables.
[f]Not used in calculation of statistics.

Table 11. Reported levels of fumaric acid (mg/100 g) in prepared juice samples

	Apple juice sample			
Lab.	A	B	C	D
11	0.73	1.18	0.30	0.74
12	1.1	1.5	0.4	0.9
15	0.91	1.54	0.48	1.16
17	0.90	1.46	0.37	0.93
19	0.93	1.46	0.41	0.93
22	0.88	1.40	0.31	0.87
27	0.46[a]	0.74[a]	0.22[a]	0.46[a]
33	0.9	1.5	0.3	0.9
35	0.5	0.9	0.5	0.6
Mean	0.856	1.368	0.384	0.879
SD	0.175	0.219	0.079	0.161
CV,[b] %	20.5	16.0	20.5	18.4
SD[c] = 0.084				
CV,[d] % = 9.7				

[a–d]See previous tables.

shows a wide variation of the precision of the results. The means for duplicate samples agree closely, but coefficients of variation for interlaboratory variability of different samples range from 6% to 79%. The data show that the mixed apple juice sample (A and D) has a value of HMF/CGA = 1.5 which is significantly higher than the expected value for authentic samples. The small standard deviations for the HMF/CGA data demonstrate that the ratio value obtained for a particular sample is sufficiently precise to detect economic adulteration of apple juice at high levels.

Malic acid determinations.—L-Malic acid content and total malic acid content in apple juice is measured because the ratio of these 2 compounds has great significance in the detection of economic adulteration. Apple juice normally contains between 150 and 910 mg/100 g of L-malic acid (6); and no D-malic acid should be present. But natural L-malic acid is much more expensive to manufacture (and purchase) than synthetic malic acid, which is a 50/50 mixture of the D- and L-isomers. The synthetic malic acid (theoretically) contains only 50% of the L- (or naturally occurring) isomer; in other words, the ratio of L-malic acid to total malic acid is 0.50 for the synthetic malic acid, compared with a 1.0 ratio for natural apple juice. If synthetic malic acid has been added to authentic juice to achieve the proper level of total acidity, we would expect the ratio of L-malic acid to total malic acid to be between 0.5 and 1.0 for such an adulterated apple juice. The lower the value of this ratio for a particular juice, the more synthetic acid has been added to the product.

ficients of variation are 4.5% or less. For this study, the participating laboratories were requested to follow a specific method for the analysis of total malic acid, which resulted in a significant reduction of the analytical variability from that obtained in a previous study where methodology was not specified (unpublished data).

Results for L-malic acid are shown in Table 9. The precision of the data was quite good with coefficients of variation in the range of 8.2% or less, and with means of duplicate samples agreeing quite closely.

Table 10 contains the calculation of the ratio of L-malic to total malic acid from data in Tables 8 and 9. The summary statistics indicate coefficients of variation for this data range from 3.4 to 8.9%.

It can be seen that the mean of the malic acid ratio for the authentic sample is very close to 1.0 and the mean for the synthetic sample comes near 0.5 as was predicted earlier. The mean for the sample mixture comes about halfway between these 2 values. The data in this table can give us an indication of the minimum level of synthetic malic acid that could be added to authentic apple juice and still be detected. From the summary statistics, using the average value obtained for the authentic juice (mean = 0.96) and bracketing it by twice the standard deviation (SD = 0.03), which for this data set is 0.06, it can be calculated that the expected ratio for an authentic sample could range from 0.90 to 1.0 (at the 95% confidence level). Any ratio value obtained which is less than 0.9 would likely have been derived from a nonauthentic juice sample. This value of the acid ratio of 0.9 is equivalent to an addition of 20% synthetic malic acid in the unknown juice sample before we could be certain (at the 95% confidence level) of adulteration.

Fumaric acid.—One additional indicator our laboratory is currently investigating is fumaric acid. This acid is a minor contaminant in synthetically produced malic acid and is readily detectable in very minute quantities by using LC. It has been suggested by Junge and Spadinger (2) that quantities of fumaric acid in apple juice of greater than 3 mg/L indicate the addition of synthetic malic acid, although this figure may have to be revised upward for juice made from concentrate.

The variability of the fumaric acid data is shown in the summary statistics of Table 11, which indicate that the interlaboratory coefficients of variation are 20% or less at the 1 mg/100 g level. It is readily observed that the synthetic juice contains much larger amounts of fumaric acid than does the authentic sample.

Conclusion

This study indicates that variability of results among laboratories depends on the method, but it can be small for experienced laboratories. The interlaboratory variabilities found in this study show that the lowest amount of adulteration that could be confidently detected is about 20%. Newer methodologies, or refinements of the current ones, could improve this figure. We now plan to subject these methods to an AOAC collaborative study.

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Symposia

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Chromatography of Amino Acids and Determination of Amino Acid Composition of Nutritionally Important Protein Sources

Chairman: Charles W. Gehrke, University of Missouri College of Agriculture

VITAMINS AND OTHER NUTRIENTS

Determination of Total Dietary Fiber in Foods and Food Products: Collaborative Study

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A collaborative study was conducted to determine the total dietary fiber (TDF) content of food and food products, using a combination of enzymatic and gravimetric procedures. The method was basically the same as published earlier (*J. Assoc. Off. Anal. Chem.* (1984) 67, 1044–1052), with changes in the concentration of alcohol and buffers, time of incubation, sample preparation, and some explanatory notes, all with the intent of decreasing the coefficient of variation (CV) of the method. Duplicate blind samples of soy isolate, white wheat flour, rye bread, potatoes, rice, wheat bran, oats, corn bran, and whole wheat flour were analyzed by 9 collaborators. TDF was calculated as the weight of the residue minus the weight of protein and ash. CV values of the data from all laboratories for 7 of the samples ranged from 1.56 to 9.80%. The rice and soy isolate samples had CV values of 53.71% and 66.25%, respectively; however, each sample contained only about 1% TDF. The enzymatic-gravimetric method for determining TDF has been adopted official first action.

A previous interlaboratory study revealed that an enzymatic-gravimetric approach for the determination of total dietary fiber (TDF) in foods, food products, and total diets was feasible (1). The collaborative study reported here used basically the same procedure, with changes in the concentration of alcohol and buffers, length of time of incubation with the enzymes, sample preparation, and explanatory notes, all with the purpose of decreasing the coefficient of variation (CV) of the method. The study was designed according to the rules of Youden and Steiner (2).

Collaborative Study

The 9 collaborators participating in this study were analysts in food companies, universities, and government laboratories representing 5 countries. Collaborators were sent 9 duplicate blind samples for analysis: (a) soy isolate—donated by Ralston Purina Co., St. Louis, MO, and used as received; (b) white wheat flour, low extraction (0.45% ash, 12% protein)—donated by General Mills, Inc., Minneapolis, MN, and used as received; (c) rye bread—Deli-Rye (Giant Foods, Inc., Washington, DC), dried for 4 h at 80°C; (d) potatoes, instant (Giant Foods); (e) rice, enriched long grain (Giant Foods); (f) wheat bran, AACC certified food grade—purchased from AACC, St. Paul, MN, and used as received; (g) oats, quick cooking—donated by Quaker Oats Co., Barrington, IL; (h) corn bran—donated by A. E. Staley Manufacturing Co., Decatur, IL, Lot No. CFL 2601 A–G, Fine, used as received; (i) whole wheat flour, high extraction (14.0% protein)—donated by General Mills, Inc., and used as received.

The rye bread, potatoes, rice, and oats were ground to a uniform size of 350 μm in a Microjet 10 centrifugal mill (Quartz Technology, Inc., Westbury, NY). No heating of the samples occurred during this procedure.

All samples were placed in 25 mL plastic scintillation vials with screw caps, with a sample letter taped to each vial. None of the samples had > 5% fat; therefore, fat extraction was not recommended. Each sample was to be dried at 70°C in a vacuum oven, or in a 105°C air oven overnight, and stored in a desiccator until analyzed.

The collaborators were further instructed to weigh the samples to the nearest 0.1 mg and express % TDF to 2 decimal places on the data sheets provided. The formula for the calculation of % TDF was also provided to each collaborator.

The collaborators were also sent the 3 enzymes, Termamyl, amyloglucosidase, and protease, needed to carry out the study.

Total Dietary Fiber in Foods
Enzymatic-Gravimetric Method
First Action

43.A14 ***Principle***

Duplicate samples of dried foods, fat-extd if contg>5% fat, are gelatinized with Termamyl (heat-stable α-amylase), and then enzymatically digested with protease and amyloglucosidase to remove protein and starch. Four vols of EtOH are added to ppt sol. dietary fiber. Total residue is filtered, washed with 78% EtOH, 95% EtOH, and acetone. After drying, residue is weighed. One duplicate is analyzed for protein, and other is incinerated at 525° and ash is detd. Total dietary fiber = wt residue − wt (protein + ash).

43.A15 ***Apparatus***

(a) *Fritted crucible.*—Porosity No. 2 (Pyrex No. 32940, coarse ASTM 40–60 μm; or Corning No. 36060 buchner, fritted disk, Pyrex, 60 mL, ASTM 40–60 μm). Clean thoroly, heat 1 h at 525°, and soak and then rinse in H_2O. Add ca 0.5 g Celite to air-dried crucibles and dry at 130° to const wt (≥ 1 h). Cool and store in desiccator until used.

(b) *Vacuum source.*—Vac. pump or aspirator equipped with in-line double vac. flask to prevent contamination in case of H_2O backup.

(c) *Vacuum oven.*—70°. Alternatively, 105° air oven can be used.

(d) *Desiccator.*

(e) *Muffle furnace.*

(f) *Water baths.*—(1) *Boiling.* (2) *Constant temperature.*—Adjustable to 60°, with either multistation shaker or multistation mag. stirrer to provide const agitation of digestion flasks during enzymatic hydrolysis.

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The recommendation of the Associate Referee was approved by the General Referee and Committee D and was adopted by the Association. See the General Referee and Committee reports, *J. Assoc. Off. Anal. Chem.* (1985) 68, March issue.

Table 1. Collaborative results (blind duplicates) of determination of TDF by the enzymatic-gravimetric method

Coll.	Soy isolate	White wheat flour	Rye bread	Potatoes	Rice	Wheat bran	Oats	Corn bran	Whole wheat flour
1	3.18[a]	2.40	6.22	5.41[b]	0.91	42.32	10.30	85.14	10.54
	0.00	2.41	6.51	5.75	0.35	41.86	11.66	86.32	11.96
2	0.73	2.63	5.61[a]	7.30	0.42[a]	40.64	9.99	83.81[c]	12.02
	—[d]	3.08	8.07	8.08	2.18	41.41	10.93	84.04	11.49
3	1.34	2.40	6.75	6.02	0.54	42.79	11.30	86.90	13.48
	1.23	2.40	7.05	6.70	0.55	42.32	11.79	87.96	13.15
4	1.19	2.79	6.30	7.85	1.28	44.01	10.98	86.70	12.70
	1.18	2.78	6.40	6.94	1.14	41.24	10.65	86.76	12.28
5	1.17	2.90	7.02	7.19	0.74	44.38	10.87	87.21	12.62
	0.00	2.91	6.35	7.38	0.82	41.75	11.25	86.80	12.43
6	0.75	2.67	6.11	7.06	0.90	42.34	10.98	87.59	13.03
	0.78	3.02	6.44	6.81	1.42	42.19	10.29	87.84	13.62
7	2.52	3.09[b,e]	6.60	6.99	0.27	43.43	12.99[b]	88.25	12.58
	—[d]	8.42	6.34	7.15	0.77	44.31	12.48	87.89	13.15
8	2.25	3.14	6.88	7.22	1.91	43.60	11.80	87.31	13.50
	1.99	3.00	7.34	7.90	1.74	44.20	10.75	88.42	12.68
9	2.70	3.08	6.35	7.97	1.49	41.96	11.70	87.47	13.11
	1.76	2.89	6.58	7.51	1.26	43.00	11.05	87.14	12.91

[a]Cochran outlier, results used.
[b]Cochran outlier, results eliminated.
[c]Grubbs outlier, results used.
[d]Erroneous results due to filtration problems.
[e]Grubbs outlier, results eliminated.

(g) *Beakers.*—Tall-form, 400 mL.

(h) *Balance.*—Analytical, capable of weighing to 0.1 mg.

43.A16 *Reagents*

(a) *95% Ethanol.*—v/v, tech. grade.

(b) *78% Ethanol.*—Place 207 mL H_2O into 1 L vol. flask. Dil. to vol. with 95% EtOH. Mix and dil. to vol. again with 95% EtOH if necessary. Mix.

(c) *Acetone.*—Reagent grade.

(d) *Phosphate buffer.*—0.05M, pH 6.0. Dissolve 0.875 g Na phosphate dibasic, anhyd. (Na_2HPO_4) (or 1.097 g dihydrate) and 6.05 g Na phosphate monobasic monohydrate (NaH_2PO_4) (or 6.84 g dihydrate) in ca 700 mL H_2O. Dil. to 1 L with H_2O. Check pH with pH meter.

(e) *Termamyl (heat-stable α-amylase) soln.*—No. 120 L, Novo Laboratories, Inc., Wilton, CT 06897. Store in refrigerator.

(f) *Protease.*—No. P-5380, Sigma Chemical Co. Keep refrigerated.

(g) *Amyloglucosidase.*—No. A-9268, Sigma Chemical Co. Keep refrigerated.

Alternatively, a kit contg all 3 enzymes (pretested) is available from Sigma Chemical Co. Cat. No. KR-185.

(h) *Sodium hydroxide soln.*—0.171N. Dissolve 6.84 g NaOH ACS in ca 700 mL H_2O in 1 L vol. flask. Dil. to vol. with H_2O.

(i) *Phosphoric acid soln.*—0.205M. Dissolve 23.64 g H_3PO_4 ACS (85%) in H_2O in 1 L vol. flask. Dil. to vol. with H_2O.

(j) *Celite C-211.*—Acid-washed, Fisher Scientific Co.

43.A17 *Enzyme Purity*

To ensure absence of undesirable enzymatic activity in enzymes used in this procedure, run materials listed in table thru entire procedure each time lot of enzymes is changed, or at max. interval of 6 months to ensure that enzymes have not degraded.

Test Sample	Activity Tested	Sample Wt, g	Expected Rec., %
Citrus pectin	pectinase	0.1	95–100
Stractan (larch gum)	hemicellulase	0.1	95–100
Wheat starch	amylase	1.0	0–1
Corn starch	amylase	1.0	0–2
Casein	protease	0.3	0–2
β-Glucan (barley gum)[a]	β-glucanase	0.1	95–100

[a] (Biocon US Inc., Lexington, KY 40509)

43.A18 *Sample Preparation*

Det. total dietary fiber on dried sample. Homogenize sample and dry overnight in 70° vac. oven, cool in desiccator, and dry-mill portion of sample to 0.3–0.5 mm mesh. If sample cannot be heated, freeze-dry before milling. If high fat content (>5%) prevents proper milling, defat with pet. ether (3 times with 25 mL portions/g sample) before milling. Record loss of wt due to fat and/or H_2O removal and make appropriate correction to final % dietary fiber found in detn. Store dry-milled sample in capped jar in desiccator until analysis is carried out.

43.A19 *Determination*

Run blank thru entire procedure along with samples to measure any contribution from reagents to residue.

Weigh duplicate 1 g samples, accurate to 0.1 mg, into 400 mL tall-form beakers. Sample wts should not differ >20 mg. Add 50 mL pH 6.0 phosphate buffer to each beaker. Check pH and adjust if necessary. Add 0.1 mL Termamyl soln. Cover beaker with Al foil and place in boiling H_2O bath 15 min. Shake gently at 5 min intervals. Increase incubation time when number of beakers in boiling H_2O bath makes it difficult for beaker contents to reach internal temp. of 100°. Use thermometer to indicate that 15 min at 100° is attained. Total of 30 min in H_2O bath should be sufficient.

Cool solns to room temp. Adjust to pH 7.5 ± 0.1 by adding 10 mL 0.171N NaOH soln.

Add 5 mg protease. (Protease sticks to spatula, so it may be preferable to prep. enzyme soln just before use with ca 0.1 mL phosphate buffer and pipet required amt.)

Cover beaker with Al foil. Incubate 30 min at 60° with continuous agitation. Cool. Add 10 mL 0.205M H_3PO_4 soln to adjust pH to 4.5 ± 0.2. Add 0.3 mL amyloglucosidase, cover with Al foil, and incubate 30 min at 60° with continuous agitation. Add 280 mL 95% EtOH preheated to 60° (measure vol. before heating). Let ppt form at room temp. for 60 min.

Weigh crucible contg Celite to nearest 0.1 mg, then wet and redistribute bed of Celite in crucible by using stream of 78% EtOH from wash bottle. Apply suction to draw Celite onto fritted glass as even mat. Maintain suction and quant. transfer ppt from enzyme digest to crucible.

Wash residue successively with three 20 mL portions of 78% EtOH, two 10 mL portions of 95% EtOH, and two 10 mL portions of acetone. Gum may form with some samples, trapping liq. If so, break surface film with spatula to improve filtration. Time for filtration and washing will vary from 0.1 to 6 h, averaging ½ h per sample. Long filtration times can be avoided by careful intermittent suction thruout filtration.

Dry crucible contg residue overnight in 70° vac. oven or 105° air oven. Cool in desiccator and weigh to nearest 0.1 mg. Subtract crucible and Celite wt to det. wt of residue.

Analyze residue from 1 sample of set of duplicates for protein by **47.021–47.023**, using N × 6.25 as conversion factor.

Incinerate second residue sample of duplicate 5 h at 525°. Cool in desiccator and weigh to nearest 0.1 mg. Subtract crucible and Celite wt to det. ash.

43.A20 ***Calculations***

Detn of blank:

$$\text{Blank} = \text{mg blank residue} - \left[\frac{(\%\text{ protein in blank} + \%\text{ ash in blank}) \times \text{mg blank residue}}{100}\right]$$

$$\text{TDF (\%)} = \text{mg residue} - [(\%\text{ protein in residue} + \%\text{ ash in residue}) \times \text{mg residue}] - \frac{\text{blank} \times 100}{\text{mg sample (wt)}}$$

Results and Discussion

An interlaboratory study (1) had indicated that the enzymatic-gravimetric procedure for determining TDF in foods and total diets was feasible and desirable. Further discussion of this method at the 2nd International Symposium on Dietary Fiber held in Washington, DC, showed that this method was one of the 2 most widely accepted procedures for determining TDF (3). In this collaborative study we have sought to: (a) determine at what levels the method is not reproducible; (b) assess the extent of starch degradation because incomplete removal of the starch will interfere with the determination of TDF; (c) evaluate mesh size for adequacy in determining TDF; and (d) improve the CV values for the determination of TDF by clarifying directions and altering some of the concentrations of the solutions.

The results of the individual analyses are shown in Table 1. Most problems occurred with the soy isolate; 2 laboratory values were discarded because of filtration problems and 1 was a Cochran outlier (2). The Cochran outlier was used in the statistical evaluation because including the value did not significantly alter the CV. For white wheat flour, the results of Collaborator 7 were not used in the final statistical analysis of the data because the value was a Cochran and Grubbs outlier (2). The TDF values for potatoes (Collaborator 1) and for oats (Collaborator 7) were not used in the statistical analyses; both were Cochran outliers. All TDF values were used in the final statistics for rye bread, rice, wheat bran, corn bran, and whole wheat flour.

The measures of precision for determining TDF are shown in Table 2. Seven food samples that had TDF values >2.78% had CV values <10%. When the foods contained >1.5% fiber, the CV was about 66%. We have substantially reduced the CV values of the food samples compared with the interlaboratory study (1). The 2 samples with low fiber content, namely, 1.42 and 1.04% for soy isolate and rice, which have large CV values, may indeed be unimportant because of their low fiber content. On the other hand, the method seems to give good results at the 2.78% dietary fiber level (white wheat flour) or higher.

Table 2. Measures of precision for determining TDF

Sample	Average % dietary fiber	Repeatability CV, %	Reproducibility CV, %
Soy isolate	1.42	66.25	66.25
White wheat flour	2.78	5.55	9.80
Rye bread	6.58	3.94	5.29
Potatoes	7.25	5.66	7.49
Rice	1.04	45.62	53.71
Wheat bran	42.65	2.33	2.66
Oats	11.03	5.30	5.30
Corn bread	86.86	0.56	1.56
Whole wheat flour	12.57	3.67	5.92

The changes in the time of enzyme incubation, concentration of buffers and reagents, and directions for carrying out the procedure have made the TDF method more rugged.

At the Spring 1981 AOAC Workshop in Ottawa (4) and after considerable discussion, the consensus was that dietary fiber for nutritional purposes should be that fraction of the food sample that is not digestible under the conditions of the test that was to be collaboratively studied. To ensure that the enzymes being used were in fact adequate for digesting naturally occurring starches under the conditions of the test method, a procedure was established for validating the efficacy of the enzyme treatment. This validation consisted of analyzing various starches with the method and confirming the absence of significant recoverable residue. We believe that this approach is still proper.

Among the samples that had recently undergone interlaboratory study (1), both potatoes and white flour had very high starch contents, yet the enzyme digested all of the starch. Lack of homogeneity of the rice sample made the starch in this sample unavailable for hydrolysis. In the present collaborative study, the mesh size has been defined, and therefore the matrix played no role in the enzymatic procedure.

Recommendation

It is recommended that the enzymatic-gravimetric method for TDF be adopted official first action.

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Assessment of Protein Quality Methodology for Infant Formulas

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Rat bioassay was used to assess the protein quality of powdered infant formulas and to evaluate the feasibility of using modified casein diets (containing the same source and level of fat and carbohydrate contributed by the infant formulas) as reference standards. Modification of the casein diet to match the milk-based formulas caused a significant reduction in weekly protein efficiency ratios (PER) and net protein ratios (NPR) at the third and fourth weeks. Modification of the casein diet to simulate the soy-based formulas had no significant effect on NPR values; PER values were more varied. When PER and NPR values of the powdered milk-based formulas were expressed relative to the unmodified reference standard, the relative values were lower than when each matched reference was used. With few exceptions, the relative weekly PER values of the soy-based formulas were similar regardless of the standard used. The relative NPR values of the formulas had a pattern similar to the relative PER values. The data indicate that protein quality evaluation of infant formulas using rat bioassay warrants the use of matched casein reference diets for each type of formula.

Standardized methodology for measuring the protein quality of infant formulas is lacking. Currently, the most widely used technique for evaluating quality of proteins in foods is the bioassay (1–4), and the official method (1) in the United States is the protein efficiency ratio (PER). However, a number of food categories such as infant formula pose specific problems that prevent strict adherence to protein quality evaluation methodology (5, 6).

The PER method (secs **43.212–43.216** (1)) defines the limit of dietary components in the protein evaluation basal diet. The guidelines suggest that proximate analyses be used to adjust diets so that all comparisons between samples and reference material are made with diets having the same content of nitrogen, fat, ash, moisture, and crude fiber. The procedure does not describe how to make these dietary adjustments when complex foods are evaluated. One of the main problems encountered when using bioassay to evaluate infant formulas is how to deal with dietary components in the formulas that fall outside the limits of the assay. Several reports (5–8) have indicated that the composition of certain food products can influence the determination of the PER, and without care in handling and interpreting the final results, conflicting values for protein quality can be obtained. Some of the problems that infant formulas pose include high moisture, high fat, high ash, and low protein and high lactose content (5, 6).

This study was undertaken to determine the feasibility of using matched casein reference controls and nonprotein groups to evaluate the protein quality of powdered infant formulas using the PER and net protein ratio (NPR) methods. The reference and nonprotein diets were formulated to contain sources and levels of fat and carbohydrate equal to those found in the individual infant formulas.

Experimental

Protein Sources

(a) *ANRC reference casein.*—87.49% protein, N × 6.25 (Humko Chemical Division, Witco Chemical Corp., Lyndhurst, NJ).

(b) *Milk-based formula I.*—Nonfat milk, coconut and corn oils, and lactose.

(c) *Milk-based formula II.*—Nonfat milk, demineralized whey, oleo, coconut and soy oils, oleic acid, lecithin, and lactose.

(d) *Soy-based formula I.*—Soy protein isolate, coconut and corn oils, lecithin, and corn syrup solids.

(e) *Soy-based formula II.*—Soy protein isolate, soy oil, lecithin, sucrose, tapioca, and dextrin.

The protein sources were analyzed according to AOAC methods (1) for fat (sec. **16.217**), ash (sec. **16.214**), nitrogen (sec. **47.023**), and moisture (sec. **16.205**). Carbohydrate was determined by difference.

Animals

Male weanling Sprague-Dawley rats, 21–28 days old, and weighing 45–55 g, were housed in individual stainless steel cages with suspended bottoms in a room maintained at 22 ± 2°C, 50 ± 10% relative humidity, with alternating 12-h periods of light and dark.

The rats were fed a commercial rat chow for 2 days. They were then weighed and the animals at the extremes of the weight distribution curve were discarded.

Assay Groups

The rats were divided into 13 groups of 10 animals each. One group received the unmodified ANRC reference casein diet; the other groups were given the test diets (Table 1). Each diet contained 10% protein (N × 6.25). The total number of rats in each test group was the same and the average weight of rats in any one group on the day beginning an assay period did not exceed by ≥5 g the average weight of the rats in any other group.

Assay Period

The AOAC method (sec. **43.215** (1)) was followed except that the body weight of each rat was recorded at the beginning of the assay period, and on days 7, 14, 21, and 28. Diet (Table 1) and water were provided ad libitum, and food intake was recorded weekly.

Calculations

The cumulative weight gain and protein (N × 6.25) intake for each rat were calculated for 1, 2, 3, and 4 weeks. The individual rat and average group PER and NPR values were calculated weekly.

PER = weight gain of test animal/protein consumed by test animal

NPR = (weight gain of test animal + weight loss of nonprotein group)/protein consumed by test animal

Relative PER (RPER) = (PER of infant formula/average PER of reference protein) × 100

Relative NPR (RNPR) = (NPR of infant formula/average NPR of reference protein) × 100

Statistical Analyses

The average unadjusted PER and NPR values for the casein reference diets were evaluated using one-way analysis of variance; when F values were significant the individual means were compared by Duncan's multiple range test (9). The

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Table 1. Diet formulations

Diet[a,b]	Diet ingredient[c] Protein source, %	Fat source, %	Sucrose–cornstarch (2 + 1), %	Other carbohydrate, %
Casein, ANRC	11.43	8.00	74.57	—[d]
Casein (milk-based I)	11.43	24.09	16.12	42.36
Casein (milk-based II)	11.43	23.85	15.88	42.84
Casein (soy-based I)	11.43	16.42	36.76	29.39
Casein (soy-based II)	11.43	14.50	38.80	29.27
Milk-based I	79.62	—	14.38	—
Milk-based II	79.37	—	14.63	—
Soy-based I	58.62	—	35.38	—
Soy-based II	56.50	—	37.50	—
Nonprotein (milk-based I)	—	24.09	27.55	42.36
Nonprotein (milk-based II)	—	23.85	27.31	42.84
Nonprotein (soy-based I)	—	16.42	48.19	29.39
Nonprotein (soy-based II)	—	14.50	50.23	29.27

[a]Names in parentheses indicate the formula used to establish the fat and carbohydrate level and source. All diets contained 10% protein.
[b]Carbohydrate and fat sources and levels: milk-based I, lactose and coconut–corn oils (1 + 1); milk-based II, lactose and oleo–coconut oil or oleic acid–soy oil (1 + 1 + 1 + 1); soy-based I, corn syrup solids and coconut–corn oils (1 + 1); soy-based II, sucrose–dextrin (6 + 1) and soy oil.
[c]Each diet contained 4.0% mineral mix (Bernhart Tomarelli, ICN Nutritional Biochemicals, Cleveland, OH), 1.0% cellulose (Alphacel, ICN Nutritional Biochemicals), and 1.0% vitamin mix (Teklad Test Diets, Madison, WI).
[d]Dash denotes no addition of dietary ingredient.

Table 2. Composition (%, w/w) of infant formulas

Component	Milk formula I	Milk formula II	Soy formula I	Soy formula II
Fat	30.3	30.1	28.0	25.7
Carbohydrate	53.2	54.0	50.1	51.8
Protein	12.6	12.6	17.1	17.7
Ash	2.8	2.5	3.3	3.6
Moisture	1.1	0.8	1.5	1.2

coefficients of variation (CV) for each protein diet at each week were determined to give an indication of precision.

Results and Discussion

Composition of Infant Formulas

All diets containing protein sources were isonitrogenous (Table 1). Only matched control diets and corresponding infant formula diets were isocaloric. The dietary formulations contained fat levels ranging from 8 to 24%; the lactose content of some diets was approximately 42% of the diet. Table 2 shows the composition of the infant formulas. The protein content of the soy-based formulas was higher and the fat and carbohydrate levels were lower than the milk-based formulas.

Nonprotein Groups

One of the major criticisms of the PER method is that it does not provide a means of evaluating maintenance requirements (10, 11). The NPR method was designed to resolve this problem by using a nonprotein group. The weight loss of animals fed the nonprotein diet is considered equivalent to the maintenance requirement of the rats tested. The weight loss of the nonprotein groups used in this study is shown in Table 3. Modifications in the carbohydrate and fat level and source had no significant effect on weight loss. These results suggest that the feeding of one nonprotein diet would be adequate in determining the NPR values of infant formulas. In this study the individual matched nonprotein groups were used to calculate NPR values.

PER and NPR Values of Reference Diets

Modification of the carbohydrate and fat level and source in the casein reference diet to match the milk-based formulas caused a significant reduction in the PER values for each of the 4 weekly periods (Table 4). The PER values of the modified diets increased with time, which appears to be related to increased adaptation to the lactose-containing diets resulting in increased food consumption and better protein utilization. The average weekly food consumption of the rats fed the milk-based diets for weeks 1–4 was 41, 51, 65, and 68 g, respectively. The development of diarrhea by rats (normally associated with lactose intolerance) was observed mainly during the first week of feeding the test diets. Burnette and Rusoff (12) indicated that high-lactose products represent a special category of foods because of the adverse reactions of rats to lactose. These authors suggested that the control diet should contain a level of lactose equal to that of the test diet and that acclimation diets formulated to contain 20% lactose should precede the feeding of test diets containing more than 20% lactose. Steinke (6) concluded that dietary factors such as lactose could have a detrimental effect on PER values and could make test samples appear lower in protein than their true value.

The weekly PER pattern for the unmodified casein reference diet was similar to casein (soy-based formula I) but not casein (soy-based formula II) at weeks 2 and 3. However, at the end of the 4-week test period, the PER values of the groups were not significantly different, suggesting that one reference standard would be practical for a 4-week assay for evaluating soy-based formulas.

For all diets, the CV was greatest the first week and decreased by the end of the second week. The CV values for the third and fourth weeks were similar, but generally lowest at the fourth week. PER values for modified casein diets simulating the milk-based formulas had the greatest variability. This was mainly a result of higher variability of the weight gain of rats fed the modified casein diet simulating the milk-based formulas. The average CV values for the weight gain of this group of rats at weeks 1, 2, 3, and 4 was 68, 37, 19, and 17%, respectively. The average CV values for the other groups was 16, 17, 12, and 11%, respectively.

Modification of the casein reference diet resulted in weekly NPR values that showed a pattern similar to that of the PER values. The NPR values of the modified casein diets containing the fat and carbohydrate level and source of the milk-based formulas were significantly lower than all other groups at the third and fourth weeks. The weekly CV values for the NPR were generally lower than the CV values for the PER. The data indicate that, regardless of the rat bioassay used, ingredients in the milk-based formula had a detrimental effect on the absolute protein quality values. These data point to the need for a separate reference standard for milk-based formulas.

Comparison of RPER and RNPR Values

Table 5 shows the absolute PER and NPR values of infant formulas and a comparison of the RPER and RNPR values using the different reference standards. Since a comparison of the absolute PER and NPR values for the infant formulas would be misleading because of the effects of some of the

Table 3. Effect of dietary modifications on weight loss of rats fed nonprotein diets[a]

Diet[b]	Week	Weight loss Mean, g	SEM,[c] g	CV, %
Nonprotein (soy-based formula I)	1	10.6	1.61	47.9
	2	19.8	0.51	8.2
	3	22.4	0.64	9.0
	4	26.1	0.55	5.6
Nonprotein (soy-based formula II)	1	11.6	0.56	15.3
	2	18.8	0.42	7.0
	3	24.0	0.47	6.2
	4	25.9	0.48	5.9
Nonprotein (milk-based formula I)	1	13.7	0.45	10.4
	2	21.3	0.47	7.0
	3	26.3	1.10	13.2
	4	29.0	0.76	8.3
Nonprotein (milk-based formula II)	1	13.0	0.30	7.3
	2	20.9	0.23	3.5
	3	25.3	0.33	4.2
	4	28.0	0.42	4.8

[a]Ten rats per group.
[b]Names in parentheses indicate the formula used to establish the fat and carbohydrate level and source.
[c]Standard error of the mean.

ingredients in the formula on protein quality values, for comparative purposes all data are expressed relative to either the unmodified casein reference diet or the casein reference diets corresponding to each individual formula. All casein reference diets were given a value of 100. When both the PERs and NPRs were expressed relative to the unmodified casein reference diet, the weekly relative values for the milk-based formulas were lower than when matched casein controls were used. The largest difference was seen with the RPER values of milk-based formula II. The magnitude of difference was as high as 158 units for milk-based formula II at the first week. With few exceptions, the unmodified casein standard and the modified standards gave similar weekly rankings for the protein quality of soy-based formulas I and II and when PER values were used. The rankings of proteins using the modified and unmodified casein reference diets and NPR values had a pattern similar to that obtained with the PER values. However, the magnitude of difference between rankings using the 2 types of reference standards was much larger for the milk-based formulas. These data suggest that the testing of infant formulas using the 2 methods warrants the use of matched casein controls for each type of formula (soy-based, milk-based).

A comparison of the methods used to rank the proteins indicated that in most cases the PER method consistently underestimated the value of the infant formulas when the unmodified casein reference diet was used. When the individual reference diets were used, there were similarities and differences in the RPER and RNPR values. The RPER values for milk-based formula II were higher than the RNPR values and the RPER values for soy-based formula I were significantly lower than the corresponding weekly RNPR values. These data suggest the need for an in-depth study to clarify which method gives the most appropriate value when individual reference controls are used for testing the quality of infant formulas.

A 14-day assay to determine NPR (13) and reduction of assay time in the PER method by half or to 21 days without loss of accuracy (14–16) have been recommended. The results of the present study indicate that, regardless of the standards used, the unit change in RNPR values from 14 to 28 days was ≤6. With the exception of milk-based formula I, there were small or no increases (CV) in precision during this time. These data lend support to a 14-day NPR assay. The change in RPER values from 14 to 28 days was ≤38 units. There was a significant increase in precision from 2 to 4 weeks for the milk-based formulas. The magnitude of the RPER rankings was similar for the third and fourth weeks and there were small increases in precision. These data do not support a 2-week assay for determining the PER values of infant formulas but do support the work of researchers (14, 17) who recommend a 3-week PER assay.

Table 4. Effect of dietary modifications on protein value of the reference casein diet[a]

Diet[b]	Week	PER[c] Mean	SEM[d]	CV, %	NPR[c] Mean	SEM[d]	CV, %
Casein	1	3.7*	0.36	9.6	5.0*	0.13	7.8
	2	3.4*	0.23	6.9	4.4*	0.06	4.5
	3	3.3*	0.14	4.3	4.1*	0.06	4.5
	4	3.2*	0.13	4.1	3.8*	0.05	4.1
Casein (soy-based formula I)	1	3.6*	0.36	9.9	4.9*	0.12	7.5
	2	3.1*,†	0.29	9.2	4.2*,†	0.10	7.3
	3	3.2*,†	0.22	6.8	4.0*	0.07	5.7
	4	3.1*	0.19	6.1	3.8*	0.06	5.4
Casein (soy-based formula II)	1	3.3*	0.43	12.8	4.8*	0.12	7.8
	2	2.9†	0.33	11.3	4.0†,‡	0.09	7.5
	3	3.1†	0.16	5.2	4.0*	0.05	3.8
	4	3.2*	0.25	8.0	3.9*	0.08	6.5
Casein (milk-based formula I)	1	1.2†	0.55	45.2	4.5†,‡	0.09	6.3
	2	1.6‡	0.49	31.2	3.8‡	0.08	6.6
	3	2.0‡	0.26	12.8	3.7†	0.05	4.5
	4	2.3†	0.24	10.3	3.6†	0.06	5.7
Casein (milk-based formula II)	1	1.0†	0.76	75.7	4.4‡	0.21	7.8
	2	1.4‡	0.41	28.3	3.8‡	0.13	7.5
	3	1.9‡	0.24	12.7	3.6†	0.11	3.8
	4	2.1‡	0.16	7.9	3.4‡	0.06	6.5

[a]Ten rats per group.
[b]Names in parentheses indicate the formula used to establish the fat and carbohydrate level and source.
[c]Each dietary treatment was compared at the same week. Values with the same superscript symbol are not significantly different.
[d]Standard error of the mean.

Table 5. Comparison of RPER and RNPR data for infant formulas using different reference standards[a]

			RPER[b]				RNPR[b]		
			Ref. std				Ref. std		
Diet	Week	PER	A	B	CV[c]	NPR	A	B	CV[c]
Soy-based formula I	1	2.4	68	66	24	3.8	78	76	12
	2	2.1	69	64	14	3.4	81	78	8
	3	2.3	73	70	12	3.3	82	81	7
	4	2.4	77	74	8	3.2	86	84	5
Soy-based formula II	1	3.1	94	83	10	4.7	98	93	4
	2	2.5	85	74	16	3.7	91	84	9
	3	2.5	82	78	15	3.5	89	87	10
	4	2.6	82	80	10	3.4	87	88	9
Milk-based formula I	1	1.2	98	32	58	5.7	129	114	52
	2	1.5	96	45	25	3.6	96	84	19
	3	2.0	99	60	13	3.6	98	88	15
	4	2.1	90	64	9	3.3	92	86	9
Milk-based formula II	1	2.1	208	50	22	4.5	104	90	8
	2	2.2	154	66	20	4.0	103	91	11
	3	2.4	122	72	10	3.7	102	90	8
	4	2.4	116	75	6	3.4	102	90	5

[a]All values are relative to a casein standard given a value of 100.
[b]A = individual reference controls were used; B = unmodified casein reference control.
[c]Using the same method, the CV was the same regardless of the reference standard used. Therefore, one listing of the CVs is given for each method.

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Liquid Chromatographic Determination of Vitamin K_1 *Trans*- and *Cis*-Isomers in Infant Formula

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The normal phase liquid chromatographic (LC) method for determination of *trans*- and *cis*-isomers of vitamin K_1 (phylloquinone) in infant formula described here uses an Apex silica column, isocratic elution, and UV absorption detection at 254 nm. Vitamin K_1 is extracted quantitatively from the product matrix by pretreating the as-fed liquid with concentrated ammonium hydroxide and methanol, and then extracting it with a 2:1 mixture of dichloromethane and isooctane. The extract is cleaned up by silica open-column chromatography and concentrated for LC analysis. For *trans*-vitamin K_1, the method precision is ≤ 3.3% RSD (relative standard deviation), and the spike recovery is 98 ± 4%. For *cis*-vitamin K_1, the precision is ≤12% RSD, determined at levels near the detection limit, and the spike recovery is 95 ± 9%. The detection limit is 0.3 ng for both isomers at signal/noise = 3.

The demand for vitamin K_1 (phylloquinone) testing has been increasing, especially after legislation of the U.S. Infant Formula Act of 1980 (1), which requires determination of vitamin K_1 in all infant formulas. Previously vitamin K_1 in infant formulas was determined by the chick bioassay, using the method of Almquist (2) with modifications. The chick bioassay, however, is not practical for routine testing because it is very costly and time-consuming, and the results are quite variable with a precision of approximately 20% RSD (relative standard deviation). Therefore, a more reliable, faster, and less expensive method for vitamin K_1 testing is necessary.

Several existing vitamin K methods (3) other than the chick bioassay have been reviewed. These include the techniques of gas chromatography (GC) (4, 5), thin layer chromatography (TLC) (6), spectrophotometry (7, 8), photochemical fluorometry (9), electrochemistry (10–12), and liquid chromatography (LC) (13–25). Among these, the LC method is preferred because it has better resolution, sensitivity, precision, and speed of analysis for detecting very low levels (ppb range) of vitamin K_1 in a variety of complex infant formula matrices. The approach of radioimmunoassay was also considered but not used, because the vitamin K_1 specific binding protein (or antibody) is not available.

Several methods using liquid chromatography (LC) to determine vitamin K_1 in infant formula (14–16, 25) and other biological materials (18–22) have been reported. These methods use reverse phase LC and determine only the total vitamin K_1; however, vitamin K_1 has *trans*- and *cis*-isomers which display dissimilar biological activities. The *trans*-isomer is essentially responsible for the antihemorrhagic activity of the vitamin, whereas the *cis*-isomer has little or no such activity (3, 26). The natural form of vitamin K_1 is the *trans*-isomer, but commercially available vitamin K_1 usually contains both isomers. Therefore, it is desirable to be able to separate and determine both isomers in infant formula, especially the *trans*-isomer, which is the active component of vitamin K_1.

Shearer et al. separated *trans*- and *cis*-isomers of vitamin K_1 on a Partisil-5 silica column as part of sample cleanup before analysis by reverse phase LC (13, 14). Hiroshima et al. separated and determined both isomers in rat plasma on a Zorbax Sil silica column, using an electrochemical detector (17).

We have developed a normal phase LC method that can separate and determine both *trans*- and *cis*-isomers of vitamin K_1 in infant formulas. Vitamin K_1 is extracted quantitatively from product matrix for further cleanup and analysis. No prior degradation of the lipids in the product by a strong alkali or enzymatic hydrolysis, used by other LC methods (16, 25), is required. This paper describes the method and the results of its application to various infant formulas.

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METHOD

Apparatus

(a) *Open-column assembly.*—Bio-Rad 1.0 cm id × 30 cm standard glass Econo-column (with polyethylene bed support) fitted with 100 mL capacity polypropylene funnel in top reservoir and Luer 3-way nylon stopcock on bottom tip (Bio-Rad Laboratories, Richmond, CA).

(b) *Liquid chromatograph.*—Beckman Model 110A pump with pressure filter; Waters WISP 710B autosample injector, or Beckman Model 210 sample injection valve with 40 μL sample loop and 1 mL syringe with 22 gauge flat-tip needle for manual injection; Beckman Model 160 or Waters Model 440 absorbance detector with 254 nm filter; and Beckman strip-chart recorder (Beckman Instruments, Inc., Berkeley, CA, and Waters Associates, Inc., Milford, MA); LC analytical columns, Apex silica column (A) and Spherisorb ODS column (B), both 4.5 mm id × 25 cm with 5 μm particles (Jones Chromatography, Inc., Columbus, OH).

(c) *Yellow fluorescent lamp.*—General Electric F40GO gold (General Electric Co., Richmond Heights, OH).

Reagents

(a) *Solvents.*—Methanol, dichloromethane, isooctane, acetone, isopropanol, all distilled-in-glass LC grade (Burdick & Jackson Laboratories, Inc., Muskegon, MI); water, distilled.

(b) *Chemicals.*—Concentrated ammonium hydroxide and granular anhydrous sodium sulfate are reagent grade (J. T. Baker Chemical Co., Phillipsburg, NJ).

(c) *Vitamin K_1 standards.*—Vitamin K_1 USP Reference Standard contains 10.0% *cis*-isomer (U.S. Pharmacopeial Convention, Inc., Rockville, MD). Prepare in isooctane solution at least 2 concentrations covering range of samples. Store in dark or under yellow fluorescent light.

(d) *LC mobile phases.*—(A) Isooctane–dichloromethane–isopropanol (70 + 30 + 0.02, v/v) for normal phase LC, and (B) tetrahydrofuran–methanol–water (27 + 67 + 6, v/v) for reverse phase LC. Degas both before use by ultrasonication for 10 min at atmospheric pressure.

Assay

Carry out entire assay under yellow fluorescent light. Keep samples and vitamin K_1 standards from exposure to any source of white light because vitamin K_1 degrades upon such exposure (27, 28, and unpublished data).

Sampling.—Pipet or weigh adequate amount of product into 500 mL separatory funnel. For ready-to-feed liquid products, sample size is 20.0 mL. For concentrated liquid or powder products, sample size is equivalent to 20.0 mL as-fed liquid, which is obtained by diluting or reconstituting product with water to 20 mL. For example, dilute 10.0 mL

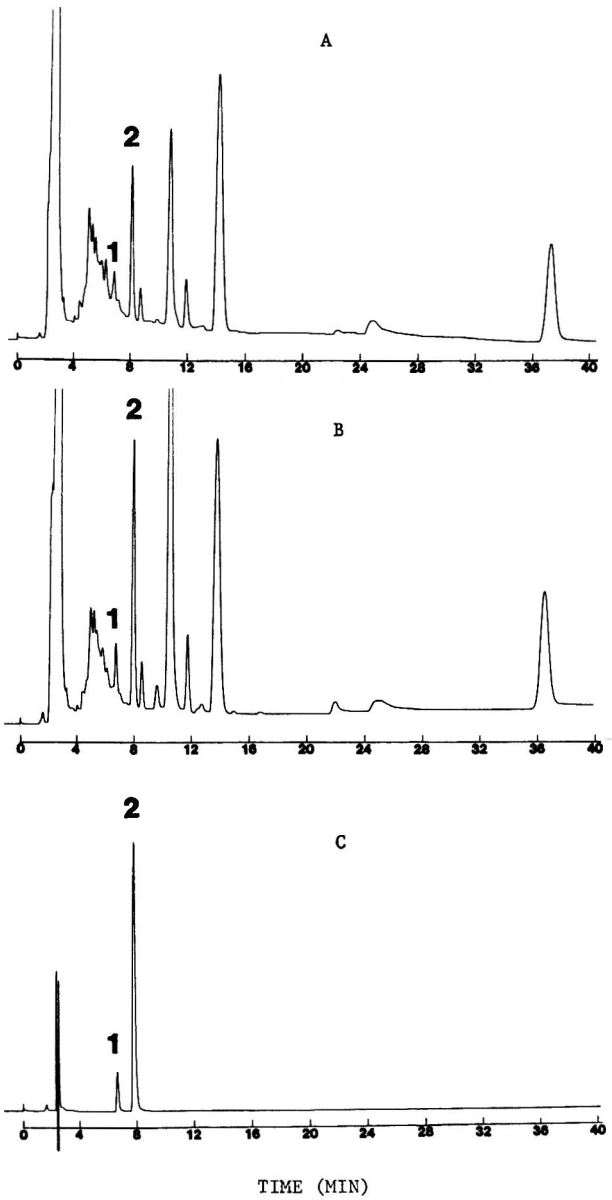

Figure 1. Typical LC chromatograms of lipid extracts of milk-based (A) and soy protein-based (B) liquid infant formulas and of vitamin K_1 standard (C); peak 1: *cis*- vitamin K_1, peak 2: *trans*-vitamin K_1. See text for chromatographic conditions.

of concentrated liquid with 10.0 mL water, or reconstitute 2.634 g powder with 18 mL water. To reconstitute powder sample, add water to funnel first and then add preweighed powder. Swirl funnel gently until all powder is well dispersed in water. Make sure that no lumps are present.

Extraction.—Add 4.0 mL concentrated ammonium hydroxide to liquid sample in separatory funnel, and swirl to mix for 60 s. Add 60 mL methanol, and swirl to mix for 30 s. Extract immediately with mixture of 100 mL dichloromethane and 50 mL isooctane. Repeat extraction once more, and combine 2 organic extracts (lower layer) in 1 L flat-bottom flask. Evaporate extract under vacuum at 70°C. Further dry oil residue by co-evaporation with 20 mL acetone 3 times, and flush with dry nitrogen. Dried oil residue should be clear.

Cleanup.—Clean up dried oil residue by silica open-column chromatography. Freshly prepare cleanup column by dry-packing 5.0 g activated (heated at 110°C overnight) silica (SilicAR cc-4, Mallinckrodt, Inc., Paris, KY) in dry 1.0 cm id × 30 cm glass open column assembly, adding 2.0 g dried (heated at 110°C overnight) granular anhydrous sodium sulfate on top of silica packing. Condition column with 20 mL isooctane containing 0.01% isopropanol. Dissolve dried oil residue in 10 mL isooctane containing 0.01% isopropanol, and load onto column. Rinse flask twice with additional 5 mL of same solvent, and load 2 rinses onto column. Rinse column wall and reservoir above packing with several portions of same solvent, totaling 10 mL. Discard all above effluent.

Quantitatively elute retained vitamin K_1 from column with predetermined amount of isooctane–dichloromethane–isopropanol (85 + 15 + 0.02, v/v), generally 100 mL. Collect eluate in 250 mL flat-bottom flask and evaporate to near dryness under vacuum at 70°C.

Determination.—Reconstitute oil residue in isooctane to 5.00 mL, and analyze it by LC using column A and mobile phase A at 1.0 mL/min (chart speed 1.0 cm/min). Set detector sensitivity to 0.005 AUFS, and use 40 or 20 μL injection volume, depending on sample concentration. Adjust flow rate, if necessary, so that retention time of *trans*-vitamin K_1 peak falls between 7.5 and 8 min. Run time is 10–11 min for standard and 42–47 min for sample.

Determine concentration of each isomer in sample by comparing peak heights with those of external standards covering concentration range of sample. Calculate as-fed concentration of each isomer in original as-fed liquid by accounting for a 4-fold concentration factor (20.0 mL to 5.00 mL). Determine total vitamin K_1 from sum of both isomers.

Column regeneration.—Analytical LC silica column used generally needs to be regenerated with sufficient (usually 180–200 mL) mobile phase after every 4 consecutive injections of sample.

Results and Discussion

This method has been applied successfully to 9 infant formulas from 4 domestic manufacturers for the determination of *trans*- and *cis*-isomers of vitamin K_1 and their total by summation, except the powder products where only the *trans*-isomer was determined. The products included both milk-based and soy protein-based ready-to-feed liquids, concentrated liquids, and powders. At least 2 lots were tested for each product, except one soy protein-based concentrated liquid where only one lot was tested. A total of 38 lots of the 9 products were tested. Seventeen lots were analyzed in quadruplicate (at least one lot from each product). The remaining lots were analyzed in duplicate, triplicate, or hexaplicate.

Chromatography

Figure 1 shows typical LC chromatograms of the lipid extracts of milk-based and soy protein-based liquid infant formulas, and that of the vitamin K_1 standard. Figure 2 shows LC chromatograms of the lipid extracts of powder products and of the standard. Chromatograms of the lipid extracts of the milk- and soy protein-based liquid products are very similar. Those of the milk- and soy protein-based powder products are also similar to each other; however, chromatograms of powder products are somewhat different from those of liquid products.

Peak identification of *trans*- and *cis*-isomers of vitamin K_1 by LC analysis was confirmed by the chromatographic behavior of both isomers on silica gel by TLC (29) and on an LC silica column (14, 17) reported in the literature, and was further confirmed by mass and proton-NMR (nuclear magnetic resonance) spectroscopic analyses of each peak collected from LC analysis.

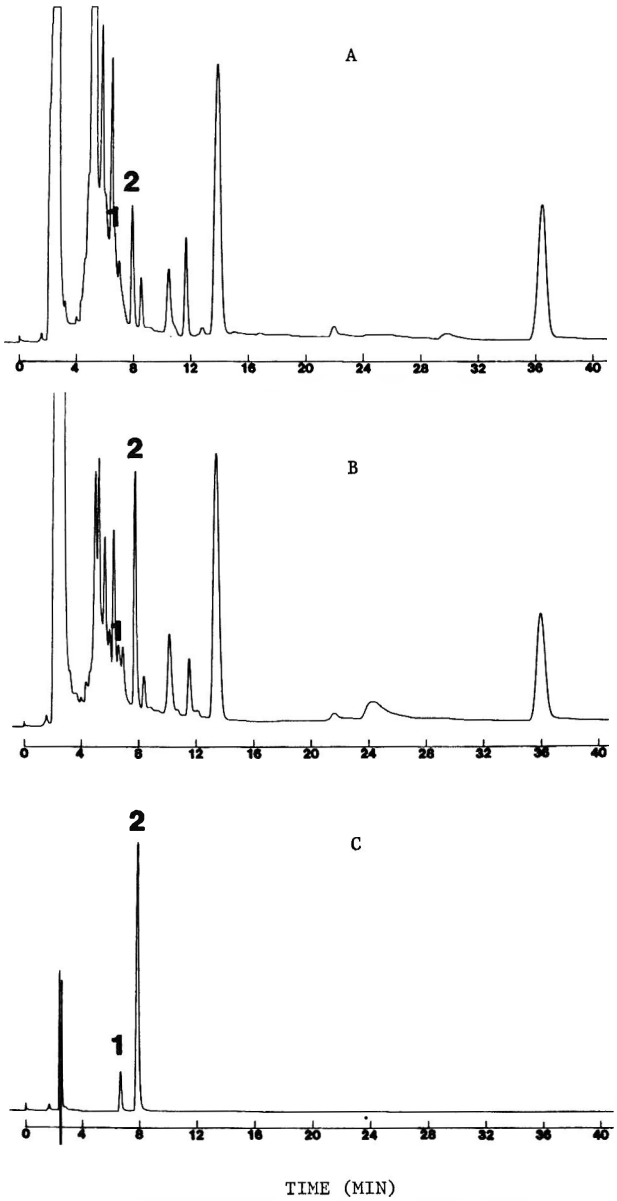

Figure 2. Typical LC chromatograms of lipid extracts of milk-based (A) and soy protein-based (B) infant formula powders and of vitamin K_1 standard (C); peak 1: *cis*-vitamin K_1, peak 2: *trans*-vitamin K_1. See text for chromatographic conditions.

In the sample chromatograms, the peak of *trans*-vitamin K_1 is well separated from the others, and thus *trans*-vitamin K_1 in all the products tested can be determined accurately. *cis*-Vitamin K_1 in the liquid products tested can also be determined, although its peak falls on the slope of a large and broad peak cluster (Figure 1). The *cis*-vitamin K_1 isomer, however, cannot be determined accurately in the powder products tested because its peak has an interference (Figure 2). This interference was later confirmed to be from corn oil, a common ingredient of the powder products.

We attempted to resolve the *cis*-isomer peak from its interference by varying the polarity of the mobile phase, but were not successful. No further effort was made because the *cis*-isomer is only a minor component and is not responsible for the biological activity of the vitamin K_1 added in most infant formulas.

Analytical Results

Table 1 shows part of the results from analysis of 9 infant formulas: one lot for product number 8, and 2 lots for each of the remaining 8 products. Each concentration (μg/L, as fed) is presented with its standard deviation, and the number of replicate analysis for each lot is shown in the last column.

Precision

The overall precision of the method for the determination of *trans*-vitamin K_1 is ≤3.3% RSD (relative standard deviation), that for *cis*-vitamin K_1 is ≤12% RSD, and that for the total vitamin K_1 is ≤4.1% RSD. These precisions were evaluated at the concentrations of *trans*-vitamin K_1 ranging from 30 to 225 μg/L, as fed, *cis*-vitamin K_1 ranging from 3 to 33 μg/L, as fed, and the total vitamin K_1 ranging from 33 to 249 μg/L, as fed. The larger uncertainty for the determination of *cis*-vitamin K_1 was caused by the low levels of the analyte near the detection limit.

Sensitivity

The linear response of peak height to concentration of the vitamin K_1 standard was confirmed up to 2800 μg/L, or 700 μg/L in the as-fed product. The detection limit of the assay at signal/noise = 3 was 0.3 ng for both isomers, equivalent to 2 μg/L of the analyte in the as-fed product, and was sensitive enough for our purpose.

Accuracy

The spike recovery of *trans*-vitamin K_1 was 98 ± 4%, that of *cis*-vitamin K_1 was 95 ± 9%, and that of total vitamin K_1 was 97 ± 4%. The spike recovery was determined from the average of 33 determinations for *trans*-vitamin K_1 at spiking levels ranging from 44 to 92 μg/L, as fed, and from the average of 26 determinations for *cis*-vitamin K_1 and total vitamin K_1 at spiking levels ranging from 5 to 12 and 49 to 102 μg/L, as fed, respectively, covering all the products tested. The results indicate that the spike recovery of the method was quantitative and the loss of vitamin K_1 during the whole assay was negligible.

Storing (refrigerated) spiked samples for up to 1 week before analysis had no effect on the recoveries. Therefore, the extraction efficiency of both the spiked and the intrinsic vitamin K_1 is believed to be quantitative. This is further supported by the findings that no more vitamin K_1 was found in the additional extracts of the remaining aqueous phase, and that more than 99% of the fat was extracted out of the sample matrix.

The loss of vitamin K_1 was further checked at each of the remaining assay steps and was found to be negligible. Even though vitamin K_1 is unstable in strong alkaline solutions (16, 27) or upon heating over 100°C (27), we found no detectable degradation under our experimental conditions.

The results of the LC method generally agreed with those of the chick bioassay (2) on replicate samples (unpublished data). However, the results of the chick bioassay were widely scattered (e.g., 34% RSD for 9 batches of products theoretically fortified with the same amount of vitamin K_1), while those of the LC method were much more consistent (e.g., 4.1% RSD for the 9 corresponding batches of products), and were within the expected range. It should be noted that the chick bioassay determines only the *trans*-vitamin K_1 and other biologically active K vitamins, e.g., K_2 vitamins.

Specificity

The peak specificity of each isomer in the chromatogram of the lipid extract was demonstrated by comparing the photodegradation rate of each isomer in the lipid extract with that in the standard, and by rechromatographing each isolated peak on a second type of column. It was shown in this labo-

Table 1. Results for LC determination of individual vitamin K_1 isomers and their total in several milk-based and soy protein-based infant formulas

Product[a]	Type[b]	Lot	Vitamin K_1, μg/L, as fed			
			trans	*cis*	Total	*n*[c]
1	RTF	1	130 ± 4	16 ± 2	146 ± 6	2
		2	138 ± 1	14 ± 1	152 ± 2	6
2	RTF	1	55 ± 1	7.0 ± 0.6	62 ± 2	2
		2	75 ± 1	3.3 ± 0.3	78 ± 1	4
3	RTF	1	30 ± 1	2.8 ± 0.2	33 ± 1	2
		2	32 ± 1	3.9 ± 0.5	36 ± 1	4
4	CL	1	132 ± 1	13 ± 1	145 ± 1	4
		2	131 ± 1	16 ± 1	146 ± 1	4
5	PWD	1	92 ± 2	—[d]	—	4
		2	90 ± 1	—	—	2
6	RTF	1	205 ± 4	25 ± 1	230 ± 5	4
		2	225 ± 2	23 ± 1	248 ± 2	3
7	CL	1	202 ± 1	27 ± 3	228 ± 4	4
		2	211 ± 1	31 ± 1	242 ± 1	2
8	CL	1	120 ± 2	7.2 ± 0.5	127 ± 2	4
9	PWD	1	175 ± 1	—	—	2
		2	195 ± 5	—	—	6

[a]Products cover 4 manufacturers and 17 different lots of commercial infant formula. Products 1–5 are milk-based and products 6–9 are soy protein-based.
[b]RTF = ready-to-feed liquid; CL = concentrated liquid; PWD = powder.
[c]Number of replicate analyses.
[d]*Cis*-Vitamin K_1 in powder products cannot be determined accurately because of peak interference.

ratory that vitamin K_1 is stable under yellow fluorescent light, but decomposed upon exposure to cool-white fluorescent light. Thus, a pooled lipid extract in a capped clear glass vial was exposed to cool-white fluorescent light at an intensity of 210 foot-candles. The remaining vitamin K_1 during the light exposure was determined by LC analysis at several time intervals: 0, 4, 8, 12, 24, and 48 h. A vitamin K_1 standard in isooctane solution was also exposed concurrently and was analyzed under the same conditions. The results showed that the degradation rates of both isomers in the lipid extract were very close to those of the corresponding isomers in the standard.

Figure 3A compares the photodegradation curve of the *cis*-vitamin K_1 in the lipid extract with that in the standard. Figures 3B and 3C compare photodegradation curves of *trans*-vitamin K_1 and total vitamin K_1 (sum of both isomers), respectively, in the lipid extract with those in the standard. Curve 1 is the degradation curve of each analyte in the standard, and curve 2 is that in the lipid extract. In each case curves 1 and 2 are almost identical. The amount of either *trans*-vitamin K_1 or the total vitamin K_1 decreases with time, while that of *cis*-vitamin K_1 increases and then decreases with time during the light exposure. The increase and then decrease of the *cis*-vitamin K_1 isomer could only be explained by isomerization of the *trans*-isomer to the *cis*-isomer, followed by decomposition of the *cis*-isomer during light exposure.

Furthermore, each isomer peak was isolated, reconstituted in isopropanol, and rechromatographed on column B, using mobile phase B (see *Method*). Each of the *trans*- and *cis*-isomer peaks isolated from either the lipid extract or the vitamin K_1 standard eluted as a single peak. All of them had the same retention time, 8 min (flow rate 1.0 mL/min), as that of the vitamin K_1 standard (*trans*- and *cis*-isomers eluting together).

Ruggedness

In the method, the volume ratio of the amount of sample (20 mL), concentrated ammonium hydroxide (4.0 mL), methanol (60 mL), and extracting solvent (100 mL dichloromethane and 50 mL isooctane) is fairly critical for quantitative extraction of vitamin K_1 from the product matrix, and for preventing the formation of foam during the extraction. The ratio of isooctane and dichloromethane of the extracting solvent is not so critical; however, use of only dichloromethane or only isooctane to extract the pretreated sample resulted in low recovery.

The silica open-column chromatographic step for cleaning the lipid extract before LC analysis is necessary. Direct injection of the lipid extract into the liquid chromatograph was tried, but the performance of the silica LC column deteriorated after one injection. The recorder baseline continued to rise and required a long time to return to an acceptable background level. With the cleanup step, the silica LC column can analyze several hundred samples without deterioration of its performance. The column, however, should be regenerated with approximately 180–200 mL mobile phase after every 4 consecutive sample injections because of a series of broad and late-eluting peaks which occur in some samples, starting at approximately 160 minutes after the injection, that would affect the analysis.

As indicated previously, 100 mL eluant was enough to quantitatively elute the retained vitamin K_1 from the silica cleanup column for all of the infant formulas tested. However, the volume of the eluant needed was shown to be affected by the amount and the type of the lipid loaded on the column. Therefore, for an unknown matrix, the necessary elution volume should be determined experimentally. Generally, the same chromatographic conditions would apply when the lipid of the sample loaded on the column weighs between 0.72 and 0.80 g. We did not use alumina in the cleanup column because vitamin K_1 was found to be decomposed on the alumina column.

Other Applications

In addition to infant formula, the method was applied to milk products, such as a commercial homogenized milk. Although no vitamin K_1 was detected (<2 μg/L), the quantitative recovery (101%) of spiked vitamin K_1 indicates the applicability of the method to this product. Either isomer of vitamin K_1 at 2 μg/L or higher in homogenized milk should be detectable. Vitamin K_1 levels in cows' milk ranging from 3.6 to 17.8 μg/L have been reported (15).

Soy oil, corn oil, and coconut oil were also analyzed by this method. The results were 1.8 ppm of *trans*-vitamin K_1 in the soy oil, 0.13 ppm in the corn oil, but less than 0.06 ppm in the coconut oil. Haroon et al. reported 1.93 ppm vitamin

K_1 in soy oil, 0.03 ppm in corn oil, and less than 0.01 ppm in coconut oil (15), while Schneider et al. reported 4.5–6.3 ppm in soy oil, 0.5–0.7 ppm in corn oil, and less than 0.1 ppm in coconut oil (30).

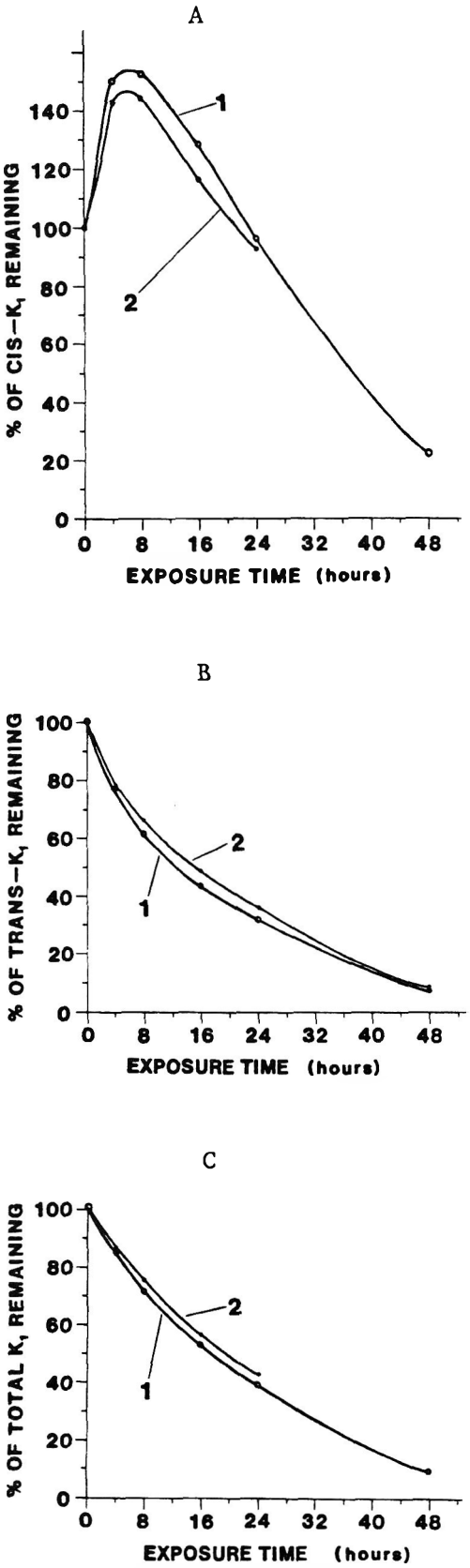

Figure 3. Photodegradation of *cis* (A) and *trans* (B) isomers of vitamin K_1 and their total (C) in the standard (curve 1) and in the lipid extract (curve 2), upon exposure to cool-white fluorescent light at intensity 210 foot-candles.

In addition, a vitamin premix and 2 commercial sources of vitamin K_1 were analyzed by diluting each sample in isooctane and directly injecting into the liquid chromatograph. The vitamin K_1 *trans*- and *cis*-isomeric composition of each sample was thus determined, and the *cis*-isomer was found to constitute 9.2–15.3% of the vitamin K_1 in these samples.

The described method is useful for the determination of *trans*- and *cis*-isomers of vitamin K_1 or their total in infant formulas not containing corn oil. Only the *trans*-isomer of vitamin K_1 can be determined accurately in those products containing corn oil. The method is more accurate and precise, and less expensive than the chick bioassay. Two assays can be completed in 8 hours. The method has also been shown to be applicable to other biological materials, with possible modifications.

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Corrected Relative Net Protein Ratio (CRNPR) Method Based on Differences in Rat and Human Requirements for Sulfur Amino Acids

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The requirement for sulfur amino acids was calculated for growing rats fed 8% protein diets. The rat and human requirements for sulfur amino acids were compared and a correction factor was developed to reflect the differences. This correction factor was used to determine corrected relative net protein ratio (CRNPR) values for a number of potential meat extenders and their mixtures with beef. The methionine + cystine requirement of growing rats was estimated to be 4% of protein for 8% protein diets. The methionine + cystine requirement of rats was about 50% higher than that of humans (2.65% of protein). Based on this comparison (rat/human), a correction factor of 1.5 was developed to correct RNPR values of those protein products that were deficient in sulfur amino acids for rat growth. The CRNPR values of beef, casein, soybean protein products, pea concentrate, and peanut meal were 100, 100, 91–97, 75, and 73, respectively. Mixtures (50:50 protein basis) of beef with casein, soybean concentrate, soybean isolate, pea concentrate, peanut meal, rapeseed concentrate, rapeseed isolate, sunflower isolate, or wheat gluten were equal to beef in CRNPR values. The CRNPR method is a good predictor of protein quality for humans of those protein products that are deficient in sulfur amino acids.

Methods for assessing protein quality of foods were recently discussed at the third session of the Codex Committee on Vegetable Proteins (1). It was concluded that the most appropriate approach for evaluating protein quality is the one based on dietary amino acids adjusted (as needed) for protein digestibility and/or amino acid bioavailability. Data on true digestibility of protein and amino acids in various foods are required to determine the nature of the digestibility adjustment(s) (1).

The desirability of correcting relative net protein ratio (RNPR, NPR of test diet expressed relative to a value of 100 for the NPR of a reference diet) to reflect differences in essential amino acid requirements of rats and humans was also suggested at the Codex Meeting (1). The RNPR method, unlike the protein efficiency ratio (PER) method, credits the protein used for both growth and maintenance. The RNPR method (2 weeks) is shorter than the standard PER method (4 weeks) and therefore less expensive, and is probably the most suitable rat assay for routine use in protein quality evaluation (2). Recent collaborative studies (3, 4) have confirmed that RNPR is more accurate and reproducible than PER. The Health Protection Branch is seriously considering replacement of PER with RNPR as the official method for regulating protein quality of foods. The adoption by AOAC of the NPR (expressed as RNPR) method as official first action as an alternative to the PER method was recommended by Happich et al. (5).

Happich et al. (5) specified the use of unsupplemented ANRC casein as the reference protein, but ANRC casein supplemented with methionine was used as the reference protein in other collaborative studies (3, 4). Diets containing 8–10% protein from unsupplemented ANRC casein were deficient in sulfur amino acids for rat growth but the methionine-supplemented (8% protein) casein diet gave the highest protein quality results and was of uniform nutritional quality (3, 4, 6).

Sarwar (7) determined amino acid-digestibility scores (corrected for true protein digestibility) and available amino acid scores (corrected for true digestibility of individual amino acids) for 17 foods and reported highly significant positive correlations ($R = 0.92$) between scores and RNPR using casein + methionine as the reference diet. However, Sarwar (7) used the FAO/WHO 1973 human pattern (8) for calculating scores, which also suits the rat amino acid requirements (9). Extensive experiments on essential amino acid requirements of 21–27 month-old children have been conducted by Pineda et al. (10) and Torun et al. (11). These estimates (11) (established by INCAP, Institute of Nutrition in Central America) for most essential amino acids were similar to values in the FAO/WHO 1973 (8) pattern, but the methionine + cystine value (2.7% of protein) was almost identical to the value (2.6% of protein) in the NRC 1980 (12) pattern. This suggested that the NRC 1980 (12) pattern is more suitable than the FAO/WHO 1973 pattern (8) for scoring proteins. Sarwar et al. (13) used the NRC 1980 pattern (12) in calculating amino acid scores, amino acid-digestibility scores, and available amino acid scores for 17 protein products. The amino acid scores, amino acid-digestibility scores, and available amino acid scores were up to 32, 30, and 19 units higher than corresponding RNPR values, respectively. The lower ratings by the RNPR compared with the available amino acid score would be caused by higher essential amino acid requirements of the growing rat compared with humans.

It is generally agreed that the requirements of rats for methionine + cystine are much higher than those of humans and that any rat growth assay will underestimate the protein quality for humans of any protein product deficient in sulfur amino acids (1). In predicting protein quality for humans by rat bioassays, modifications for higher essential amino acid requirements of rats compared with humans have been suggested (14).

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Table 1. Data used in estimating methionine + cystine requirement of the growing rat (Figure 1)

Diet	Methionine, g/100 g protein	Cystine, g/100 g protein	True digestibility, %		RNPR (casein + methionine = 100)
			Methionine	Cystine	
Lentil[a]	0.80	0.94	78	82	37
Fababean[a]	0.75	1.25	78	86	40
Pea flour[b]	1.11	1.51	77	84	54
Wheat flour + pea flour[b]	1.16	1.73	84	93	66
Wheat flour + beef[b]	1.95	1.62	89	89	79
Casein[b]	3.02	0.47	96	83	86

[a]Unpublished data. Amino acid contents were determined in duplicate by previously reported procedures (18). True digestibility (rat balance method) values were determined in duplicate on pooled feces of 8 rats/diet. RNPR data were average of 8 individual values (8 rats/diet).
[b]Methionine and cystine data (determed in duplicate by 7 collaborating laboratories) were taken from Sarwar et al. (18), true digestibility data (determined in duplicate on pooled feces of 6 rats/diet) were taken from Sarwar (7), and RNPR data (determined by 6 collaborating laboratories using mostly 8 rats/diet) were taken from Sarwar et al. (4). Samples studied in these 3 investigations were taken from the same batches.

The current NRC requirement for rat growth for total sulfur amino acids is 0.6% of the diet (9). This requirement is based on a diet containing 12% protein (having 100% true digestibility and 100% biological value) and 3.6 kcal of metabolizable energy (ME) per g of diet (assuming 95% of the digestible energy is metabolizable). Amino acid requirements for rat growth are related to dietary protein concentration and should be adjusted for caloric density of the diet (9). If expressed as percent of the diet, the requirement for an amino acid tends to increase as protein content increases. But the requirement may remain constant or decrease slightly if expressed as percent of dietary protein (9). When expressed as percent of protein, the NRC requirement for rat growth for sulfur amino acids would be 5%. Other reports have suggested that this requirement may be too high (15, 16). Peace et al. (16) estimated the methionine + cystine requirement of the growing rat at the 8% protein level and reported a value of 4.1% of protein. Pick and Meade (17) reported that the sulfur amino acid requirement of the growing rat fed 9.4% protein diets was 4.26% of protein.

The objectives of the present work were (a) to obtain more information on sulfur amino acid requirement for rat growth, (b) to develop a correction factor for correcting RNPR, based on differences in rat and human requirements for sulfur amino acids, and (c) to determine RNPR and corrected RNPR (CRNPR) values of a number of potential meat extenders and their mixtures with beef.

Experimental

The data used in estimating the methionine + cystine requirement of the growing rat are given in Table 1. Diets containing 8% protein and 3.8 kcal ME/g were used in obtaining data on true digestibility of amino acids and RNPR (casein + methionine = 100) contained in Table 1. ME was calculated by using the Atwater factors of 4, 9, and 4 kcal/g for protein, fat, and available carbohydrates, respectively. It has been shown that a diet containing 8% protein from ANRC casein plus 0.2% L-methionine meets (or exceeds) the sulfur amino acid requirement for rat growth at this protein level when NPR is used as the performance criterion (6).

Ten protein sources and their mixtures were studied in RNPR determinations. The samples of beef (lean grade minced beef, freeze-dried and defatted), ANRC casein, and rapeseed protein concentrate were obtained from the same batches studied in previous collaborative investigations (4, 18). The samples of soybean protein concentrate and pea protein concentrate were provided by Griffith Laboratories, Toronto, Ontario. Soybean protein isolate was purchased from Frank E. Dempsey and Sons, Toronto, Ontario. The samples of peanut meal and wheat gluten were purchased from Teklad Mills, ARS/Sprague-Dawley Division of the Mogul Corp., Madison, WI. The samples of rapeseed and sunflower protein isolates were supplied by the POS Pilot Plant Corp., Saskatoon, Saskatchewan.

Diets containing 8% protein (N × 6.25) from casein + 0.2% L-methionine (reference diet), beef, casein, soybean concentrate, soybean isolate, pea concentrate, peanut meal, rapeseed concentrate, rapeseed isolate, sunflower isolate, wheat gluten, or mixture (50:50 protein basis) of beef with each one of the nonmeat protein products, and a N-free diet were included in the rat feeding study. The N-free (or basal) diet contained (air-dry basis): corn oil, 10.0%, alphacel, 1.0%, mineral mixture (19), 3.5%, vitamin mixture (20), 1.0%, and cornstarch, 85.5%. Each of the protein sources or mixtures was added to the basal diet to provide 1.3% dietary N (or 8% protein) at the expense of cornstarch. The protein diets were made isonitrogenous by the addition of L-glutamic acid.

The 21 diets were fed to male weanling (54–63 g) CD Sprague-Dawley rats in a randomized complete block design using 8 blocks (based on initial body weights) of 21 rats as reported previously (4). Prior to the start of the feeding study, rats were kept in stock cages and fed a commercial rat chow diet for 2 days. Rats were then allocated into groups of 8 having approximately the same mean weights.

During the test period, rats were housed individually in stainless steel screen-bottom cages at 24–25°C and 40–50% humidity. Food and water were provided ad libitum for 4 weeks (2 weeks for the N-free diet), and records of weekly weight gains and food consumption were kept.

NPR and RNPR values (2 weeks) were calculated by using the following formulas:

$$\text{NPR} = (\text{wt gain of test animal} + \text{wt loss of nonprotein animal})/(\text{protein consumed by test animal})$$

Table 2. Comparison of human and rat requirements for essential amino acids

Amino acid, % of protein	Rat[a]	Human[b]	Rat/human ratio
Isoleucine	4.17	3.65	1.14
Leucine	6.25	7.15	0.87
Lysine	5.84	5.75	1.01
Methionine + cystine	4.00	2.65	1.51
Phenylalanine + tyrosine	6.67	7.10	0.94
Threonine	4.17	3.60	1.16
Tryptophan	1.25	1.17	1.07
Valine	5.00	4.30	1.16

[a]Values for all amino acids except methionine + cystine for rat growth were taken from NRC (9). Value for methionine + cystine was that estimated in present investigation. Arginine (5.00% of protein) and histidine (2.50% of protein) are also essential for rat growth (9).
[b]Values were average of INCAP (11) and NRC 1980 (12) reference patterns. Latter pattern (12) also includes a requirement for histidine (1.70% of protein).

Table 3. NPR and RNPR values of beef, some potential meat extenders, and their mixtures with beef

Protein sources	NPR	RNPR
Casein + Met (control)	5.60	100
Beef	5.12	91
Casein	4.65	83
Soybean concentrate	3.66	65
Soybean isolate	3.43	61
Pea concentrate	2.82	50
Peanut meal	2.72	49
Rapeseed concentrate	4.86	87
Rapeseed isolate	4.59	82
Sunflower isolate	2.06	37
Wheat gluten	1.75	31
Beef + nonmeat products:		
Casein	4.82	86
Soybean concentrate	4.27	76
Soybean isolate	4.11	73
Pea concentrate	4.03	72
Peanut meal	4.11	73
Rapeseed concentrate	4.94	88
Rapeseed isolate	4.84	86
Sunflower isolate	4.36	78
Wheat gluten	4.34	77

NPR values for each diet were calculated by averaging values for all 8 blocks.

$$\text{RNPR} = (\text{NPR of test diet}/\text{NPR of ref. diet}) \times 100$$

Moisture and nitrogen were determined by AOAC (1980) procedures (21). Samples were hydrolyzed with 6N HCl, performic acid + 6N HCl, and 4.2N NaOH, and amino acids were determined by ion exchange chromatography using a Beckman 121MB analyzer (18).

Results and Discussion

RNPR (casein + methionine = 100) data (Table 1) were plotted against available methionine + cystine (total amino acid × true amino acid digestibility, as determined by rat balance method) contents (Table 1) in estimating the methionine + cystine requirement for rat growth (Figure 1). A straight line was fitted to the points ($R = +0.99$) and the line was extended. The methionine + cystine requirement level for rat growth was considered achieved when RNPR reached 100. According to this approach, the methionine + cystine requirement for rat growth (at the 8% protein level) was estimated to be 4% of protein (Figure 1). This confirmed previous studies (15, 16) which suggested that the current NRC requirement (9) for rat growth for sulfur amino acids (5% of protein) is rather high. The higher NRC-specified requirement is to make allowance for the active role of methionine as a methylating agent over the minimum needed for protein synthesis (9). Since the vitamin mixture used in the present study provided an adequate level (0.1%) of dietary choline, the requirement for methionine as a methyl donor may have been reduced.

Essential amino acid requirements of humans and rats are compared in Table 2. The rat requirement for methionine + cystine was about 50% higher than that of humans (Table 2). The differences between the requirements for other essential amino acids were relatively small. Based on these comparisons (Table 2), a factor of 1.5 was chosen to correct the RNPR of protein products deficient in sulfur amino acids for rat growth. The use of the correction factor would be appropriate in predicting protein quality (CRNPR) for adults and children but not for infants because the amino acid requirements of infants may be different (higher) than those used for adults in Table 2 (2, 12).

The samples of beef, casein, soybean concentrate, soybean isolate, pea concentrate, peanut meal, rapeseed concentrate, rapeseed isolate, sunflower isolate, and wheat gluten tested in this investigation contained 95.2, 94.7, 70.2, 92.2, 57.0, 61.2, 68.3, 87.3, 92.7, and 87.0% protein (N × 6.25, dry basis), respectively. NPR and RNPR values of beef, the 9 potential meat extenders, and their mixtures with beef are shown in Table 3. None of the nonmeat protein products was equal to beef in RNPR ratings (Table 3). The RNPR values of the rapeseed protein products, 82–87, were higher than those for other vegetable protein products. Wheat gluten had the lowest RNPR of 31. The RNPR values of the soybean protein products, pea concentrate, peanut meal, and sunflower isolate were 61–65, 50, 49, and 37, respectively. In most cases, addition of beef substantially improved RNPR values (Table 3). The beneficial effect of the addition of beef was most noticeable in the case of wheat gluten and sunflower

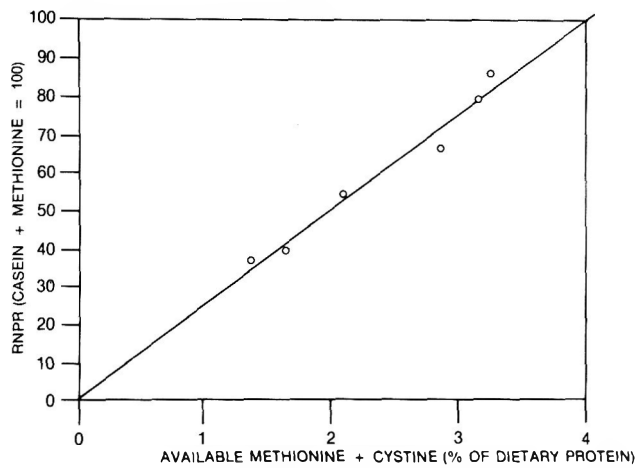

Figure 1. Estimation of methionine + cystine (corrected for true digestibility, available) requirement of growing rats fed 8% protein diets, using RNPR as performance criterion.

Table 4. Amino acid composition (g/16 g N) of protein products studied in this investigation[a]

Product	Ile	Leu	Lys	Met + Cys	Phe + Tyr	Thr	Trp	Val
Beef[b]	4.18	7.75	7.94	3.27	7.02	4.21	1.03	4.54
Casein[b]	5.36	10.16	8.44	3.49	11.50	4.64	1.31	6.85
Soybean concentrate	4.40	7.60	6.40	2.60	9.00	3.80	1.80	4.90
Soybean isolate	4.64	8.00	6.02	2.34	8.92	3.82	1.33	4.85
Pea concentrate	4.27	7.67	7.68	2.62	8.49	4.17	0.87	4.96
Peanut meal	3.23	6.05	3.19	2.11	8.09	2.45	0.78	3.55
Rapeseed concentrate[b]	4.16	7.84	5.70	4.43	7.06	4.19	1.50	5.24
Rapeseed isolate	4.51	7.79	5.07	3.62	7.52	4.42	1.44	5.30
Sunflower isolate	4.34	6.32	2.24	2.50	8.49	3.23	1.38	5.06
Wheat gluten	3.23	6.29	1.50	3.30	7.55	2.34	0.96	3.58

[a]Abbreviations for amino acids: Ile = isoleucine, Leu = leucine, Lys = lysine, Met = methionine, Cys = cystine, Phe = phenylalanine, Tyr = tyrosine, Thr = threonine, Trp = tryptophan, Val = valine.

[b]Data for beef (all amino acids except tryptophan), casein, and rapeseed concentrate were taken from Sarwar et al. (18). Beef was re-analyzed for tryptophan content in present investigation.

Table 5. Essential amino acids provided by protein sources expressed as % of rat requirements for amino acids (% of protein) as noted in Table 2[a]

Protein sources	Ile	Leu	Lys	Met + Cys	Phe + Tyr	Thr	Trp	Val
Casein + Met	128	164	145	150	172	110	109	137
Beef	99	125	137	82[b]	105	100	86	91
Casein	128	164	145	87	172	110	109	137
Soybean concentrate	105	123	110	65	134	90	150	98
Soybean isolate	110	129	104	58	133	91	111	97
Pea concentrate	102	124	132	65	127	99	72	99
Peanut meal	77	98	55	53	121	58	65	71
Rapeseed concentrate	99	126	98	111	105	100	125	105
Rapeseed isolate	107	126	87	90	112	105	120	106
Sunflower isolate	103	102	39	62	127	77	115	101
Wheat gluten	77	101	26	82	113	56	80	72
Beef + nonmeat products:								
Casein	114	144	141	84	138	105	97	114
Soybean concentrate	102	124	124	73	120	95	117	94
Soybean isolate	105	127	120	70	119	95	98	94
Pea concentrate	100	124	135	73	116	100	79	95
Peanut meal	88	111	95	67	113	79	75	81
Rapeseed concentrate	99	126	118	96	105	100	105	98
Rapeseed isolate	103	125	112	85	108	103	102	98
Sunflower isolate	101	113	88	72	116	89	100	96
Wheat gluten	88	113	81	74	109	78	82	85

[a]Abbreviations for amino acids are defined in Table 4.
[b]First limiting amino acid is underlined.

Table 6. CRNPR values of products limiting in sulfur amino acids for rat growth

Product	CRNPR[a]
Beef	100
Casein	100
Soybean concentrate	97
Soybean isolate	91
Pea concentrate	75
Peanut meal	73
Beef + nonmeat products:	
Casein	100
Soybean concentrate	100
Soybean isolate	100
Pea concentrate	100
Peanut meal	100
Rapeseed concentrate	100
Rapeseed isolate	100
Sunflower isolate	100
Wheat gluten	100

[a]The CRNPR values >100 were considered as 100.

isolate, in which the RNPR values were more than doubled (Table 3). The supplementary effect of beef noted in most cases can be attributed to the higher lysine or methionine + cystine contents of beef than those of vegetable protein products (Table 4). This suggested that in regulating nutritional adequacy of extended meat products, the requirement for protein quality (and quantity) should be placed on the final extended product and not on the extender. This method will make allowance for supplementary effect of meat in meat-vegetable protein mixtures on protein quality.

To obtain information on limiting amino acids in test proteins for rat growth, essential amino acids provided by protein sources (Table 4) were expressed as a percent of rat requirements (Table 5). The samples of beef, casein, soybean concentrate, soybean isolate, pea concentrate, peanut meal, and all mixtures with beef were most limiting (or first limiting) in methionine + cystine (Table 5). The remaining 4 products (rapeseed concentrate, rapeseed isolate, sunflower isolate, and wheat gluten) were most limiting in lysine. These calculations (Table 5) did not include corrections for true digestibility of individual amino acids. Such corrections would be required to accurately predict the first limiting amino acid especially in those cases where 2 or more amino acids are equally limiting. Most of the products tested in this investigation were clearly limiting in either methionine + cystine (casein, soybean concentrate, soybean isolate, and all mixtures of beef with nonmeat products except rapeseed protein concentrate) or lysine (sunflower isolate and wheat gluten) (Table 5). The differences between the first and second limiting amino acids in the remaining 6 products such as beef, pea concentrate, peanut meal, rapeseed concentrate, rapeseed isolate, and beef + rapeseed concentrate were not as large (less than 10 percentage units). However, the corrections (not shown here) for true digestibility of amino acids in the 6 products (mostly unpublished data) did not change the nature of amino acid deficiency.

The RNPR values (Table 3) of the protein products deficient in sulfur amino acids for rat growth (Table 5) were multiplied by the factor of 1.5 to obtain CRNPR values (Table 6). The CRNPR values for beef or casein and the soybean protein products were 100 and 91–97, respectively. The samples of pea concentrate and peanut meal had CRNPR values of 75 and 73, respectively. All mixtures of beef + nonmeat products were equal to beef or casein in CRNPR ratings (Table 6).

The CRNPR data suggested that soybean concentrate was comparable to beef or casein while soybean isolate was about 10% lower in protein quality when compared with beef or casein (Table 6). The slightly higher CRNPR value of soybean concentrate over soybean isolate could be attributed to the marginally higher methionine + cystine content of the former product (Tables 4 and 6). Recent studies with young men have demonstrated that the protein quality of soybean isolate is comparable to that of milk or beef (22–24).

Unlike RNPR and other rat growth assays for assessing protein quality, the CRNPR method would not discriminate against proteins with lower sulfur amino acid content. The correction factor of 1.5 developed in this investigation (to reflect differences between sulfur amino acid requirements of humans and rats) can be refined as new information on amino acid requirements of humans and rats becomes available. Further studies are required to determine the need for developing similar correction factors for other essential amino acids. The practical application of the correction factor developed in this investigation would be limited to those protein products that have been shown to be first limiting in sulfur amino acids for rat growth. Some examples of such protein sources include soybean protein products, peanuts, and grain legumes or pulses, i.e., peas, beans, and lentils (25–30).

Acknowledgments

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Improved Liquid Chromatographic Determination of Riboflavin in Milk and Dairy Products

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Reported here is a simple liquid chromatographic (LC) method for the determination of riboflavin in milk (liquid, evaporated, and dry), yogurt, and cheese. The method involves passing liquid samples or filtrates of semisolid and solid samples through a C_{18} cartridge. Retained riboflavin is then eluted with an aliquot of 50% methanol in 0.02M acetate buffer of pH 4. A volume of the eluate is injected into the LC system consisting of a C_{18} column, a solvent of water–methanol–acetic acid (65 + 35 + 0.1, v/v) with a flow rate of 1 mL/min, and a UV detector set at 270 nm. The method is precise and accurate and compares favorably with the present AOAC method. Moreover, it involves fewer sample preparation steps and has a total analysis time of less than 1 h.

The present AOAC method for the determination of riboflavin in milk and dairy products (1) is time consuming and involves many sample preparation steps. Moreover, the sample is treated with oxidants to remove background fluorescence, and this may destroy riboflavin (2). Another fluorometric method for the determination of riboflavin in milk was developed (3); the method is simple and rapid, but it lacks specificity.

Several liquid chromatographic (LC) methods for the determination of riboflavin in foods have been reported (4–6). These methods are more specific than fluorometric methods because they separate riboflavin from interfering compounds before quantitation; however, these methods involve considerable sample preparation.

In a previous study (6), we developed an LC method to determine riboflavin in eggs and dairy products. In this report, we describe an improved LC method for riboflavin determination in dairy products, which is simpler and more rapid.

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Experimental

Apparatus

(a) *Liquid chromatograph.*—Waters Associates equipped with Model 6000A pump, Model U6K injector, and data module (Waters Associates, Milford, MA 01757).

(b) *LC column.*—250 × 4 mm Bio-Sil ODS-5S C_{18} column (Bio-Rad Laboratories, Richmond, CA 94804).

(c) *Detector.*—UV, Gilson Model 222 (Gilson Medical Electronics, Middleton, WI 53562), set at 270 nm, and sensitivity unit of 0.02.

Reagents

(a) *Water.*—Pass distilled, deionized water through 0.45 μm filter membrane.

(b) *Methanol.*—Anhydrous, chromatography grade.

(c) *LC mobile phase.*—Water–methanol–glacial acetic acid, analytical grade (65 + 35 + 0.1).

(d) *Acetate buffer.*—0.02M, pH 4.0.

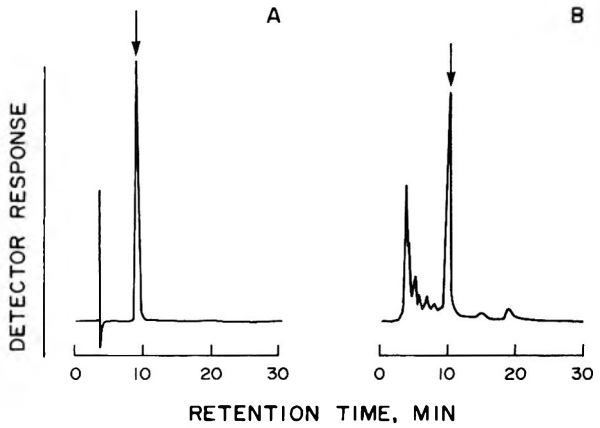

Figure 1. LC chromatograms of standard riboflavin (A), and whole milk (B). Riboflavin peak is marked by arrow and represents 46.5 and 40 ng, respectively. See text for chromatographic conditions.

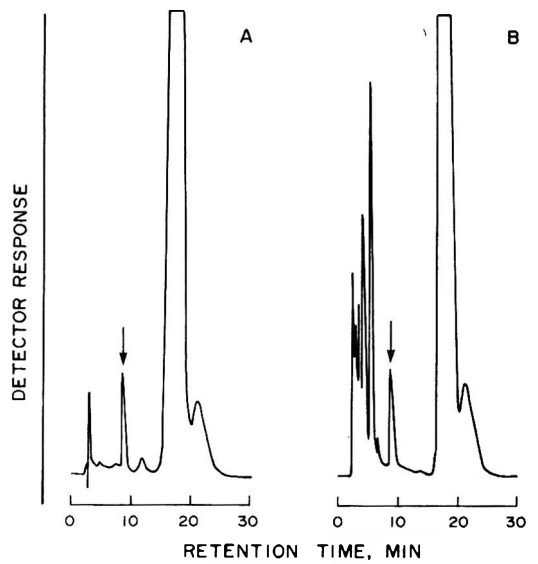

Figure 2. LC chromatograms of plain yogurt (A), and cottage cheese (B). Riboflavin peak is marked by arrow. See text for chromatographic conditions.

(e) *Eluting solution.*—Mix equal volumes of acetate buffer (d) and methanol.

(f) *Riboflavin standard solution.*—5 μg/mL. Accurately weigh 5 mg riboflavin (anhydrous, Sigma Chemical Co., St. Louis, MO 63178), and dissolve, by stirring, in 900 mL acetate buffer in 1 L volumetric flask covered with aluminum foil. When riboflavin is completely dissolved, dilute to volume with acetate buffer, mix well, and refrigerate.

Sample Preparation

Protect samples from light during preparation. Cover tubes and flasks with aluminum foil and work under subdued lighting conditions.

(a) *Skim milk, 2% fat milk, and whole milk.*—Warm refrigerated milk samples to room temperature. Connect C_{18} cartridge (Sep-Pak, Waters Associates) to 30 mL glass syringe with metal Luer-Lok tip (VWR Scientific, Norwalk, CA 90650). Activate cartridge by passing through 5 mL methanol followed by 5 mL water. Pipet 10 mL milk sample into syringe, then pass through cartridge. Wash cartridge twice with 10 mL water and discard washings. Pipet 5 mL eluting solution (e), pass through cartridge, and receive eluate in screw-cap tube. Mix thoroughly, cap tube, and refrigerate until LC analysis. Reactivate cartridge for next sample.

(b) *Evaporated milk.*—Mix equal volumes of evaporated milk and water. Pipet 10 mL of mixture into syringe and pass through cartridge. Proceed as in (a).

(c) *Dry milk.*—Stir 10 g dry milk with 10 mL water in beaker. After complete suspension, transfer quantitatively to 100 mL volumetric flask and dilute to volume with water. Pipet 10 mL milk into syringe and pass through cartridge. Proceed as in (a).

(d) *Dairy products.*—Blend appropriate weight (25 g yogurt, 25 g cottage cheese, 10 g cheddar cheese, 10 g Monterey Jack cheese, 10 g process cheese) with 70 mL 0.02M acetate buffer for 5 min in Waring Blendor at low speed (adjust speed with variable transformer, VWR Scientific, if necessary to minimize heat). Filter blend quantitatively through Whatman No. 4 paper, and receive filtrate in 100 mL volumetric flask. Dilute to volume with 0.02M acetate buffer, and mix thoroughly. Pass 25 mL of filtrate through activated cartridge (in small portions). Proceed as in (a).

LC Analysis

Set detector at 270 nm and sensitivity at 0.02. Set flow rate at 1 mL/min. Bring refrigerated riboflavin standard solution and sample eluate to room temperature. Inject 10 μL of riboflavin standard solution into LC system. Establish retention time and peak area, and calibrate data module accordingly (external standard method). Inject 25 μL of sample eluate into LC system, and record amount of riboflavin from calibrated data module. Calculate riboflavin content in μg/mL for liquid samples or μg/g for semisolid and solid samples. Recheck data module calibration at regular times by injecting 10 μL of riboflavin standard solution into LC system.

Recovery Study

Add proper volume of riboflavin standard solution to sample so that riboflavin content of spiked sample is approximately doubled. Prepare spiked sample, and determine its riboflavin content as described above. Calculate percent recovery.

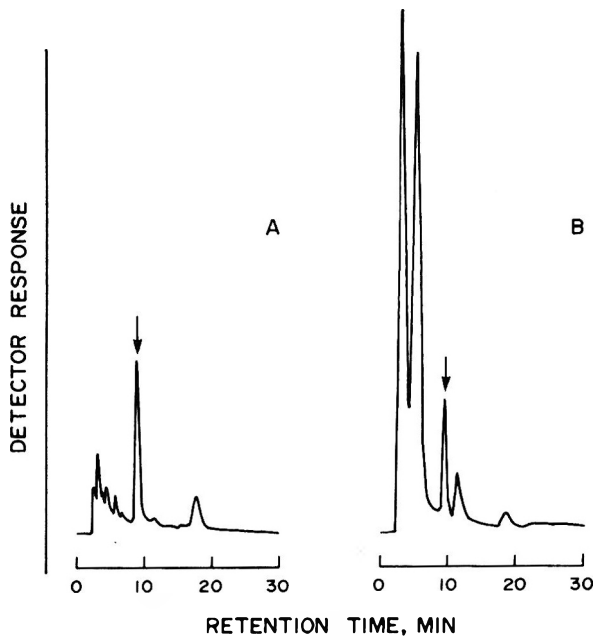

Figure 3. LC chromatograms of process cheese (A) and cheddar cheese (B). Riboflavin peak is marked by arrow. See text for chromatographic conditions.

Table 1. Effect of heating on riboflavin content of milk and dairy products

Sample	Riboflavin content[a]	
	Unheated sample	Heated sample
Whole milk	1.28 ± 0.05	1.11 ± 0.07
Nonfat dry milk	16.4 ± 0.7	15.1 ± 0.6
Yogurt (plain)	0.56 ± 0.03	0.47 ± 0.02
Process cheese	1.68 ± 0.08	1.54 ± 0.02
Cheddar cheese	2.51 ± 0.04	2.00 ± 0.07

[a]Average of 6 determinations ± SD, μg/mL milk, μg/g other samples.

Table 2. Riboflavin content of milk and dairy products

Sample	Riboflavin content[a]	CV, %
Skim milk	1.25 ± 0.02	1.6
2% Fat milk	1.24 ± 0.07	5.6
Whole milk	1.28 ± 0.05	3.9
Evaporated milk	2.53 ± 0.10	4.0
Nonfat dry milk	16.4 ± 0.7	4.3
Yogurt (plain)	0.56 ± 0.03	5.4
Cottage cheese	0.56 ± 0.02	3.6
Process cheese	1.68 ± 0.08	4.8
Monterey Jack cheese	1.52 ± 0.09	5.9
Cheddar cheese	2.51 ± 0.04	1.6

[a]Average of 6 determinations ± SD, μg/mL for first 4 samples, μg/g others.

AOAC Method

The riboflavin content of the dairy products was also determined by the AOAC official method (1). The results obtained by the AOAC method were then compared with those of this LC method.

Preparation of Heated Samples

To study the effect of heating on riboflavin content, selected dairy samples were heated under the conditions stated in the AOAC method (1). The heated samples were then cooled to room temperature, filtered through Whatman No. 4 paper, and treated as in *Sample Preparation*. The riboflavin content of the heated samples determined by this LC method was compared with that of identical samples without the heat treatment.

Results and Discussion

The LC conditions used in this study for the separation and quantitation of riboflavin in milk and dairy products were similar to the conditions we reported previously (6). The percent methanol in the solvent system was increased in this study to 35% to shorten analysis time. Under the present LC conditions, the riboflavin peak is resolved at a retention time of 9.4 min with no interference from other milk components, as shown in Figures 1, 2, and 3. The identity of the riboflavin peak was confirmed by spiking or coinjecting samples with known amounts of standard riboflavin. Only the riboflavin peak increased proportionally upon spiking or coinjecting.

The present AOAC method for the determination of riboflavin (1) calls for acidifying milk and dairy products with 0.1N HCl to less than pH 4.5, and then autoclaving at 121–123°C for 30 min. Proteins are precipitated, and bound riboflavin is liberated by such treatments. However, when milk and dairy products were acidified and heated according to the AOAC method, there was no increase in riboflavin content when compared with unacidified and unheated identical samples, as shown in Table 1. These results indicate that the riboflavin content of milk and dairy products is not affected by acidification and heating. They confirm our previous findings (6) and those of others (3).

Table 2 shows the riboflavin content of milk and selected dairy products obtained by this LC method. These data show that the method is versatile and precise, with coefficients of variation (CV) between 1.6 and 5.9%. Recoveries of riboflavin from spiked samples by this LC method ranged from 94.1 ± 0.23 to 100.7 ± 3.2, with CV values of 0.24 to 5.9% (Table 3). These results indicate that the method is also accurate.

The riboflavin content of milk and dairy products analyzed by this LC method and by the AOAC method compares favorably, as shown in Table 4.

In addition to the specificity, precision, and accuracy of the described LC method, it is also simple and convenient. It involves minimal preparation steps and requires short analysis time. In our previous method (6), proteins in milk and dairy products were first precipitated by acidification, and then removed by centrifugation. In this LC study, there was no need to precipitate milk proteins in any of the samples analyzed. Currently, the average riboflavin analysis time for a milk sample is 20–25 min, and that of the dairy products is 30–45 min. Some dairy products yielded late peaks not present in milk (Figures 2 and 3), which necessitated longer analysis time. None of the samples analyzed, however, required analysis time longer than 1 h under the conditions used.

Another advantage of the present LC method over other LC methods (4–6) is that the eluate injected is free of milk proteins, because none of the milk proteins are retained on the C_{18} cartridge with riboflavin. This results is much lower column back-pressure, fewer column washings, and longer column life.

It is essential to test the efficiency of the C_{18} cartridge by passing through 5 mL of riboflavin standard solution, eluting, and quantitating riboflavin. The cartridge should not be used when the efficiency is lower than 95%, i.e., less than 95% of riboflavin is retained. In our laboratory, one C_{18} cartridge is used for 5 milk samples or 3 dairy product samples before being discarded. Regeneration of the used C_{18} cartridges will be investigated in the future.

Table 3. Recovery of riboflavin from spiked samples by LC method

Sample	Rec., %[a]	CV, %
Skim milk	99.0 ± 3.3	3.3
2% Fat milk	94.6 ± 5.6	5.9
Whole milk	95.1 ± 2.3	2.4
Evaporated milk	94.1 ± 0.23	0.24
Nonfat dry milk	97.8 ± 2.8	2.9
Yogurt (plain)	100.7 ± 3.2	3.2
Monterey Jack cheese	94.5 ± 5.2	5.5
Cheddar cheese	96.8 ± 3.0	3.1

[a]Average of 6 determinations ± SD.

Table 4. Riboflavin content of milk and dairy products by LC and AOAC methods

Sample	Riboflavin content[a]		CV, %	
	LC[b]	AOAC[c]	LC	AOAC
Skim milk	1.25 ± 0.02	1.06 ± 0.01	1.6	0.94
Whole milk	1.28 ± 0.05	1.27 ± 0.02	3.9	0.79
Evaporated milk	2.53 ± 0.10	2.27 ± 0.06	4.0	2.6
Nonfat dry milk	16.5 ± 0.7	15.5 ± 0.7	4.3	4.5
Yogurt (plain)	0.56 ± 0.03	0.68 ± 0.04	5.4	5.9
Cottage cheese	0.56 ± 0.02	0.54 ± 0.02	3.6	3.7
Process cheese	1.68 ± 0.08	1.89 ± 0.03	4.8	1.6
Cheddar cheese	2.51 ± 0.04	2.60 ± 0.02	1.6	0.77

[a]μg/mL for the first 3 samples, μg/g for others.
[b]Average of 6 determinations ± SD.
[c]Average of 2 determinations ± SD.

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Symposia

at the 99th AOAC Annual International Meeting

October 27-31, 1985 — The Shoreham Hotel — Washington, DC

Quality Assurance

Chairman: Susan Glad Andersen, Hazleton Laboratories America

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Immunotoxicity

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Chromatography of Amino Acids and Determination of Amino Acid Composition of Nutritionally Important Protein Sources

Chairman: Charles W. Gehrke, University of Missouri College of Agriculture

EXTRANEOUS MATERIALS

Extraction of Light Filth from Whole Peppermint Leaves: Collaborative Study

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Collaborators: C. L. Cox; W. L. Davis, Jr; J. LaGreca; R. W. Potter; M. C. Stricklin; F. C. Zimmerman

Results are reported for a collaborative study of a method for the extraction of light filth from whole peppermint leaves. A 5 g sample is defatted with isopropanol in a simple reflux appartus. Rat hairs, insect fragments, and whole insects are isolated by wet sieving on a No. 230 sieve, a deaerating boil in 40% isopropanol solution, flotation with Tween 80–Na_4EDTA (1 + 1) and mineral oil–heptane (85 + 15), and trappings in a Wildman trap flask. Average recoveries obtained by 6 collaborators for 3 spike levels of rat hairs (5, 10, 15) were 83.3, 87.5, and 82.2%, respectively. For whole insects (5, 10, 15) recoveries averaged 85.0, 80.0 and 77.2% respectively; for insect fragments (20, 30, 50) recoveries averaged 79.6, 88.3, and 84.8%, respectively. The average recoveries for the 3 levels of each analyte were not significantly different. The method has been adopted official first action.

Peppermint belongs to a large group of botanicals for which there are no official methods for separating filth. These products usually consist of plant parts such as flowers, leaves, seeds, and roots, which are ground and blended for teas or used for flavorings and the production of extracts. Initial investigations centered on common leafy botanicals. This collaborative study involves only whole leaf peppermint. The proposed method can also be performed satisfactorily on other botanicals, i.e., alfalfa, lemon balm, papaya, and spearmint. The proposed method will be collaboratively studied with these products in the future.

The method involves defatting a 5 g sample with isopropanol in a simple reflux apparatus (Dent, R. G. (1982) *J. Assoc. Off. Anal. Chem.* **65**, 1089–1092) for 10 min. Rat hairs, insect fragments, and whole insects are isolated by wet sieving on a No. 230 sieve, a deaerating boil in 40% isopropanol solution, flotation with Tween 80–Na_4EDTA (1 + 1) and mineral oil–heptane (85 + 15), and trappings in a Wildman trap flask. A standing period of 5 min following the magnetic stirring of the flotation liquid and vigorous agitation of flask contents at intervals during the flask fill aided the recoveries of whole insects.

Collaborative Study

Three levels of analyte spike were used in this study. The low level consisted of 5 rat hairs (2.5–5 mm), 20 insect fragments (elytral squares of *Tribolium* sp., ca 0.5 sq. mm), and 5 whole insects (adult *Lasioderma serricorne*, cigarette beetle). The intermediate level was 10 rat hairs, 30 insect fragments, and 10 whole insects; the high level was 15 rat hairs, 50 insect fragments, and 15 whole insects. The spike was placed in randomly numbered vials, and 2 vials of each analyte level were sent to each of 6 collaborators, together with 6 preweighed samples of product. The collaborators were instructed to report their analysis times and to return the extraction papers so that their results could be checked by the Associate Referee.

Light Filth in Whole Peppermint Leaves
Flotation Method
First Action

44.A06 ***Apparatus and Reagents***

(a) *Simple reflux apparatus.*—**44.125(e)**.
(b) *Tween 80–40% isopropanol.*—**44.003(x)**.
(c) *Tetrasodium EDTA–40% isopropanol.*—**44.003(z)**.

44.A07 ***Pretreatment***

Add 5 g sample and 500 mL isopropanol to 1 L beaker. Boil 10 min with mag. stirrer, using reflux app.

44.A08 ***Isolation***

Wet-sieve product on No. 230 sieve with hot H_2O until washings are clear. Quant. transfer residue to 2 L trap flask with 40% isopropanol. Fill flask to 400 mL with 40% isopropanol. Boil on hot plate for 10 min with slow mag. stirring, **44.002(n)**. Cool to 20–25° in H_2O bath. Add 100 mL premixed Tween 80–Na_4EDTA (1 + 1) down stirring rod. Hand-stir gently 1 min. Let stand 5 min. Fill flask to 800 mL with 40% isopropanol. Add 50 mL flotation liq., **44.003(k)**, down stirring rod. Mag. stir, **44.005(c)**, 5 min. Let stand 5 min. Fill flask successively to 1200 mL, 1600 mL, and into neck with 40% isopropanol down stirring rod, agitating contents vigorously (up-down) with rod and wafer for 3–4 s after each fill. Let stand 5 min. Again vigorously agitate flask contents. Let stand 25 min. Trap into 400 mL beaker, washing neck, wafer, and rod with 40% isopropanol and add rinse to beaker. Add 35 mL flotation liq. Hand-stir (up-down) for 30 s. Let stand 20 min. Trap into original beaker, washing neck, wafer, and rod with isopropanol and add rinse to beaker. Filter onto ruled filter paper, wash beaker with isopropanol, and filter washings. Examine papers microscopically at 30×.

Results and Discussion

In no instance was excessive plant material evident on filter papers returned to the Associate Referee. The collaborators took an average of 2.8 h/subsample to perform the extraction procedures (range 2–4 h) and an average of 0.5 h/subsample to count the plates (range 0.125–1.25 h).

Comparisons of mean percentage recoveries for experimental data with ranges from the collaborative study (Tables 1–3, Associate Referee counts) demonstrate good agreement; these recoveries are 81 vs 82–88% for rat hairs, 95 vs 77–85% for whole insects, and 82 vs 80–88% for insect fragments.

Most of the collaborators obtained good recoveries of rat hairs (Table 1). Collaborator A had consistently low recoveries that ranged from 50 to 60%, for which no explanation could be found. The recoveries reported by Collaborator E also appeared to be consistently low, but a check of the plates showed that a number of hairs had not been counted. It was learned that Collaborator E based the counts on what was believed to be the predominant hair size, rather than on the size range given in the collaborative study instructions. The actual recoveries for rat hairs are given in parentheses in Table 1. Correcting the counts substantially reduced the apparent variation in the recovery data.

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Table 1. Collaborative results for recovery of rat hairs (blind duplicates)

Coll.	Spike level 5		10		15	
A	4(3)[a]	3	5	5	9(8)	7(8)
B	5	5	10	11	15(13)	14
C	9(4)	6(5)	9(10)	11(10)	14	14(15)
D	5	4(5)	10	8(9)	12	12(10)
E	2(4)	3(4)	6(8)	9(11)	10(13)	6(12)
F	6(5)	2	9	7	14(15)	15(14)
$\bar{x}$	4.5(4.17)		8.33(8.75)		11.83(12.33)	
$\bar{x}$, %	90.0(83.33)		83.33(87.5)		78.89(82.22)	
SD						
Repeatability	1.53(0.91)		1.35(1.12)		1.35(0.82)	
Reproducibility	2.02(1.04)		2.21(2.17)		3.25(2.57)	
CV, %						
Repeatability	34.0(21.82)		16.21(12.8)		11.41(6.65)	
Reproducibility	44.89(24.94)		26.53(24.8)		27.47(20.84)	

[a]Associate Referee counts are in parentheses if different from those of collaborator.

Table 2. Collaborative results for recovery of whole or equivalent insects (blind duplicates)

Coll.	Spike level 5		10		15	
A	6(5)[a]	9(5)	8	17[b](10)	13(12)	14(9)
B	5	2	5	9(10)	9(8)	10(9)
C	1(2)	5	6	5(6)	8(7)	15
D	5	4	8	9	14	14
E	5(4)	5	10(9)	9(8)	15(14)	5(6)
F	4(5)	4	8	8(9)	15(16)	15
$\bar{x}$	4.58(4.25)		7.73(8.0)		12.25(11.58)	
$\bar{x}$, %	91.67(85.0)		77.27(80.0)		81.67(77.22)	
SD						
Repeatability	1.66(1.32)		1.28(1.63)		3.55(3.4)	
Reproducibility	1.66(1.32)		1.71(1.63)		3.55(3.57)	
CV, %						
Repeatability	36.24(31.06)		16.56(20.38)		28.98(29.36)	
Reproducibility	36.24(31.06)		22.12(20.38)		28.98(30.83)	

[a]Associate Referee counts are in parentheses if different from those of collaborator.
[b]Outlier by Dixon test; not included in calculations.

The recoveries of whole or equivalent insects (W/E), although somewhat variable, were generally acceptable (Table 2). Because Collaborator A considered any major body part, such as a thorax–abdomen or an abdomen, to be an equivalent insect, the counts were considerably inflated. As defined in the collaborative study instructions, an equivalent insect consists of at least a head. The corrected counts are in parentheses.

Collaborators B and C had consistently low recoveries of W/E insects. However, both had noted additional W/E insects rising to the interface following the second (final) trapping. Collaborator B also stated that, because no specific time was given for the intermittent agitation during the flask filling, this operation was performed for 30, 30, and 45 s, respectively. The duration for these steps should have been only a few seconds to simply agitate the flask contents. Excessive agitation at these points could have shaken insects from the flotation liquid.

No significant problems were noted with insect fragment (IF) results (Table 3). The data are in agreement with the consistent recoveries noted in the ruggedness test results for IFs (Table 4).

Finally, Student's *t*-tests were performed to compare percentage recoveries for levels of each analyte (Tables 1–3). No significant differences were found ($P > 0.05$).

Recommendation

On the basis of the satisfactory analyte recoveries and clean filter papers, it is recommended that the proposed method be adopted official first action.

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Table 3. Collaborative results for recovery of insect fragments (blind duplicates)

Coll.	Spike level 20		30		50	
A	9	8	23	27(28)	40(35)	46(48)
B	18(19)[a]	20(19)	27(26)	27	40	38
C	19(17)	9	19(20)	24(23)	37	31
D	20	18	30	30	50	47(50)
E	19(17)	18(19)	31(28)	26(28)	43(45)	39
F	21(19)	17	26(27)	27(28)	47(49)	46(47)
$\bar{x}$	16.33(15.92)		26.42(26.5)		42.0(42.42)	
$\bar{x}$, %	81.67(79.58)		88.06(88.33)		84.0(84.83)	
SD						
Repeatability	3.24(2.53)		2.36(1.73)		2.92(4.56)	
Reproducibility	4.88(4.63)		3.4(3.13)		5.63(6.69)	
CV, %						
Repeatability	19.84(15.89)		8.93(6.53)		6.95(10.75)	
Reproducibility	29.88(29.08)		12.87(11.81)		13.4(15.77)	

[a]Associate Referee counts are in parentheses if different from those of collaborator.

Table 4. Ruggedness test data for extraction of light filth[a] in whole peppermint leaves: comparison of variations

Operation as performed in proposed method	Variation 1 Operation	Rec.,[b] % RH	IF	W/E	Variation 2 Operation	Rec.,[b] % RH	IF	W/E	Signif. diff.[c] in recoveries
Solvent saver boil, 10 min	10 min	81	75	90	25 min	75	86	68	no
Hot plate boil, 10 min	9 min	74	75	75	15 min	82	86	82	no
Cool to 20–25°C	15–19°C	79	81	90	26–30°C	78	80	68	no
Magnetically stir, 5 min	4 min	79	76	65	8 min	78	85	92	no
Let stand 5 min	5 min	81	85	82	10 min	75	76	75	no
Vigorous stirring during flask filling	no stirring	81	75	72	vigorous stirring	75	86	85	no

[a]RH = rat hairs; IF = insect fragments; W/E = whole or equivalent insects.
[b]Four replications (20 RH, 20 IF, 10 W/E).
[c]Between variations 1 and 2 ($P > 0.05$).

Determination of Internal Insect Infestation of Oats: Collaborative Study

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An improved method has been developed for determining internal insect infestation of oat kennels. The method involves alcohol defatting and acid hydrolysis of the cracked oats, wet sieving to remove the acid, transfer to a 2 L Wildman trap flask, deaeration by boiling, and treatment with Tween 80–Na_4EDTA. Insects are extracted with light mineral oil. Reports from 6 collaborators showed that recoveries averaged 88.98% for adult insect heads and 97.22% for larvae. The method has been adopted official first action.

The present official AOAC method for internal insect infestation in grain (1) produces an excessive amount of plant material which floats and is trapped off with the heptane layer. As a result, 30–35 readable extraction papers are needed per sample, and in some samples, the trapped plant material cannot be filtered. For these reasons, the present AOAC method is unsatisfactory for oats.

In the proposed method, a 25 g sample is used. The sample is cracked, treated with isopropanol to dissolve the plant waxes, and sieved while the isopropanol and dissolved waxes are removed with water. The material is then hydrolyzed with HCl to break down the starch. The hydrolyzed sample is placed on a sieve and washed with water to remove the acid to prevent any chemical reaction between HCl and Na_4EDTA. The material is then transferred to a 2 L trap flask with 40% isopropanol, deaerated, and treated with Tween 80–Na_4EDTA solution to prevent the plant material from floating with the oil. Mineral oil is added and the contents are mixed. Because the trapped-off oil layer contains very little plant debris, 2 clean filter papers are produced for counting.

In the method reported by Trauba (2), the grain is cracked by using a Labconco mill, or equivalent, or an electric coffee grinder set to give a particle size of about 0.061 in. The Norelco "dial-a-bean" coffee grinder, mentioned in the method, is no longer manufactured. Other coffee grinders of this type are available and should be satisfactory for cracking the grain.

The particle size of the cracked oats, about 0.061 in., can be checked by passing the 25 g of cracked oats through a No. 14 sieve (3). The amount of cracked oats retained on the sieve should range from 75 to 80% by weight, and the amount passing through the sieve should range from 20 to 25% by weight.

When the Labconco mill is used, the blade-adjusting wheel should be rotated counterclockwise until the cutting blades are touching. Then, the adjusting wheel should be rotated clockwise ¾ of a revolution. This setting should give cracked oats within the desired particle size range.

Collaborative Study

Six collaborators each examined six 100 g spiked samples of previously cracked oats by the official method and the same number of spiked 25 g samples by the proposed method. The spikes in all samples consisted of the adult heads of 15 *Sitophilus oryzae* and 15 *Rhyzopertha dominica*, and 5 whole *Sitophilus oryzae* larvae.

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Insect Infestation (Internal) in Oats

Cracking Flotation Method

First Action

44.A01 ***Reagent***

Tween 80–Na_4EDTA [(ethylenedinitrilo)-tetraacetic acid tetrasodium salt] premix soln.—Measure 420 mL 40% isopropanol in 500 mL graduate. Add 80 mL Tween 80 (polysorbate 80) to 100 mL g-s graduate. Invert 100 mL graduate over 2 L glass beaker and drain briefly. Rinse 100 mL graduate with several portions of the 420 mL 40% isopropanol, pouring each rinse into beaker. Add rest of 40% isopropanol to beaker, add mag. stirring bar, **44.002(n)**, and start mag. stirring. Add 10 g Na_4EDTA to beaker while stirring rapidly. Add 500 mL 40% isopropanol and stir until uniform. Mixed reagent stored in g-s flask is stable 1 week.

44.A02 ***Preparation of Sample***

Mix grain by passing 6 times thru Jones sampler, recombining sepns before each pass. Sep. slightly >25 g and weigh 25 g. Transfer weighed sample, small amt at a time, to 5 or 8 in. No. 12 sieve, and with stiff bristle brush, work insects thru sieve as completely as possible.

Grind screened sample in cutting-type mill set at 0.061 in. (Elec. coffee grinder can be used.) Particle size of cracked oats, ca 0.061 in., can be checked by passing the 25 g cracked oats thru No. 14 sieve, **44.002(r)**. Amt of cracked oats retained on sieve should range from 75 to 80% by wt and amt passing thru sieve should range from 20 to 25% by wt. When using Labconco mill, rotate blade adjusting wheel counterclockwise until cutting blades are touching. Then rotate adjusting wheel clockwise ¾ revolution. This setting should give cracked oats within particle size range mentioned above. Dry damp or tempered grain in forced-draft oven 1 h at 70–80° or 2 h in oven without draft.

44.A03 ***Isolation***

Transfer cracked grain, including any residue in mill, to 2 L glass beaker contg mag. stirring bar, **44.002(n)**, and add 600 mL isopropanol. Cover beaker and stir *gently* while boiling 5 min on hot plate.

Transfer sample to No. 100 sieve, **44.002(r)**, with *gentle* stream of hot tap H_2O. Wash material on sieve with *very gentle* stream of hot (55–70°) tap H_2O to remove isopropanol. Quant. transfer material on sieve to original 2 L beaker with H_2O. Add mag. stirring bar, **44.002(n)**, and mixt. of 600 mL H_2O + 50 mL HCl. Cover beaker and mag. stir contents *gently* while boiling 15 min on hot plate. Transfer material to No. 100 sieve, **44.002(r)**, with *gentle* stream of hot tap H_2O. Wash material on sieve with *very gentle* stream of hot (55–70°) tap H_2O until washings show no acidity when tested with blue litmus paper.

Add mag. stirring bar, **44.002(n)**, to 2 L trap flask, **44.002(h)**(*4*). Place wide-stem funnel in flask opening and quant. transfer material on sieve to flask with 40% isopropanol. Add 40% isopropanol to total vol. of 800 mL.

Clamp stirring rod so stopper or wafer is above liq. in flask. (Trap flask may stand overnight at this point.) Stir *gently* while boiling 7 min ± 10 s on mag. stirring hot plate. Remove flask from hot plate and wash down sides with min. of 40% isopropanol and immediately add 100 mL Tween 80–Na_4EDTA soln slowly down rod. Hand-stir *gently* 1 min and let stand 3 min.

Table 1. Collaborative results for recovery of adult insect heads (30 added) and larvae (5 added) from oat kernels by the proposed method

	Adult heads						Larvae					
Coll.	A	B	C	D	E	F	A	B	C	D	E	F
1	21	29	29	26	25	26	4	5	5	5	5	4
2	29	19	25	27	28	25	5	5	5	4	5	5
3	28	22	23	29	28	25	5	5	5	5	5	4
4	30	30	29	29	20	28	5	5	5	5	5	5
5	27	27	25	27	27	29	5	5	5	5	5	5
6	28	30	27	28	28	28	5	4	5	5	5	5
Av., %			88.98						97.22			
SD												
Repeatability			2.808						0.357			
Reproducibility			2.808						0.357			
CV, %												
Repeatability			10.52						7.34			
Reproducibility			10.52						7.34			

Add 50 mL mineral oil, **44.003(p)**, down stirring rod. Stir mag., **44.005(c)**, 5 min on cool mag. stirrer, and let stand 3 min.

Fill flask with 40% isopropanol, added slowly down stirring rod to avoid mixing or agitation of flask contents, and let stand 20 min *undisturbed*. Trap off into beaker, rinsing neck of flask with 40% isopropanol, and add rinse to trapping in beaker.

Transfer trapping to ruled filter paper, rinsing beaker well with isopropanol. Add 35 mL mineral oil, **44.003(p)**, to flask and hand-stir 1 min. Clamp stirring rod so stopper or wafer is at midpoint of flask. Let stand 5 min, spin stirring rod to free settlings from stopper or wafer, and adjust oil level with 40% isopropanol to ca 1 cm above fully raised stopper. Let stand *undisturbed* 15 min. Trap off into beaker, rinsing neck of flask well with isopropanol and adding rinsings to trapping in beaker. Transfer trapping to ruled filter paper, rinsing beaker well with isopropanol. Examine papers at 15×, counting only whole insects, insect heads, cast skins, and head capsules.

Results and Discussion

Table 1 shows the recoveries of the spiked elements. Average recoveries of adult insect heads and larvae by the proposed method were 88.98 and 97.22%, respectively. The method gave good average recoveries and precision for the adult insect heads and larvae and 2 clean, easy-to-read extraction papers.

The official method was determined to be unsatisfactory for oat kernels because of the excessive amount of plant material that was trapped off with the heptane layer. From 30 to 35 readable extraction papers were needed per sample, and in some samples the trapped material could be filtered. For these reasons, the collaborators were instructed not to make counts of the extraction papers from the official method. Therefore, no recovery results for the official method are presented.

Recommendation

The proposed method produces clean extraction papers and high recoveries for both adult and larval whole or equivalent insects. No toxic solvents, which need special precautions, are used. Therefore, it is recommended that the proposed method for the determination of internal insect infestation of oat kernels be adopted official first action.

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(1) *Official Methods of Analysis* (1984) 14th Ed., AOAC, Arlington, VA, sec. **44.043**
(2) Trauba, R. L. (1981) *J. Assoc. Off. Anal. Chem.* **64**, 1408–1410
(3) *Official Methods of Analysis* (1984) 14th Ed., AOAC Arlington, VA, sec. **44.002(r)**

OILS AND FATS

Mass Spectrometry and Identification of Sterols in Vegetable Oils as Butyryl Esters and Relative Quantitation by Gas Chromatography with Flame Ionization Detection

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Electron ionization mass spectrometry (MS) of sterol butyrates is described. Fragmentation of common sterol butyrates is related to structure and is discussed in relation to the fragmentation of free sterols and of commonly used sterol derivatives. Derivatized samples of vegetable oils are introduced using a 10 m capillary gas chromatographic (GC) column for complete separation of the sterol butyrates. Quantitation of sterol butyrates in vegetable oils by packed column GC/flame ionization detection is based on percent relative area of peaks identified by MS. Results of analyses of sunflower, castor, rapeseed, and virgin olive oils, and other oils are presented. These techniques have been applied to the rapid screening of marketed olive oils for possible adulteration.

Sterols are the principal component of the unsaponifiable lipid fraction of vegetable oils. However, they constitute only about 1% of the total lipids. The major lipid fraction, the saponifiable fraction, makes up about 95% of the lipid and consists of triacylglycerols (1).

Many papers have been published on the quantitative and qualitative sterol composition of vegetable oils. Itoh et al. (2) reported a detailed analysis of Spanish olive oils and Fedeli (3) reviewed the lipid content of various olive oils. Mannino and Amelotti (4) determined the sterol content of a variety of vegetable oils. The sterol fraction of edible oils has been suggested for use in identifying the oil and detecting adulteration (1, 3, 5, 6). Standard methods of sterol determination include extraction, thin layer chromatography (TLC), and gas chromatography (GC) of the trimethylsilyl (TMS) ether derivatives (2, 7).

The most common 4-desmethylsterols found in vegetable oils include cholesterol (trace levels), brassicasterol, campesterol, stigmasterol, β-sitosterol, and Δ^5-avenasterol (1–3). In addition, the 4-methyl- and 4,4-dimethylsterols have been characterized in vegetable oils (1–3). Itoh et al. (8) continued the application of GC to sterol determination, using sterol acetates. Acetate and other derivatives are often used to facilitate GC and to avoid peak tailing. The use of butyryl esters of sterols was described for the determination of sterols in food (9) based on the work of Nelson et al. (10). Sterol butyrates provided improved GC resolution compared to other derivatives.

GC/mass spectrometry (MS) has been extensively applied to the characterization of plant sterols (1–3). The electron ionization (EI) MS of sterols (11, 12) and other steroidal compounds (13–16) has been described. Knights (17) compared the EI fragmentation of Δ^5, Δ^7 and diunsaturated sterols and their acetyl, trifluoroacetyl, and TMS ether derivatives. Under EI conditions, sterols and certain of these derivatives produce a molecular ion and in all cases afford characteristic fragment ions that can be correlated with structure.

In this paper we describe the EI MS of sterol butyrates. Fragmentation is related to structure and compared with the fragmentation of free sterols and their commonly used derivatives. Sterol butyrates are identified by MS in derivatized samples of vegetable oils by using a 10 m capillary GC column to completely separate the major sterol constituents. Quantitation of sterols as butyrates is based on percent relative area and is achieved by packed column GC/flame ionization detection (FID). Results of analyses of several vegetable oils are presented, and application of these techniques to the screening of olive oils for adulteration is discussed.

Experimental

Apparatus and Reagents

(a) *Gas chromatograph-mass spectrometer.*—Finnigan-MAT (San Jose, CA) 3300F quadrupole mass spectrometer operated under the following conditions: electron energy 70 eV; emission current 0.50 mA; preamplifier and multiplier 10^{-8} A/V and −1400 V. The electron multiplier detector was modified to include conversion dynode/multiplier (Finnigan No. 4007-60210/Galileo No. 4752) similar to that used on Finnigan 4000 series instrument. Additional feed-through for the dynode high voltage was provided by using the cup feed-through connector for the signal lead and the normal signal feed-through for the dynode high voltage, which was operated at −3 kV.

Finnigan 9500 gas chromatograph was operated under the following conditions: injector 240°C; column temperature held at 40°C for 1 min following injection and then ballistically elevated to 300°C; separator and transfer lines held at 250°C. The capillary column (10 m, SE 54, fused silica, Hewlett-Packard, Avondale, PA) was directly connected to the ion source by removing the glass jet separator and using a 1/16–1/4 in. adaptor (No. LHR4/16, Scientific Glass Engineering (SGE), Austin, TX). The original packed column injector was modified first by replacing it was a splitless injector (SGE No. SCI-AK) and then modifying it as follows: The glass-lined stainless steel tubing that is part of the SCI-AK injector insert and enters from the bottom of the injector was replaced with 13.4 cm glass tubing made of joined 3 and 4 mm id glass. This modified glass injector insert then overlaps both the glass-lined tubing left in the insert and also the neck of the septum purge head (SGE No. PH8 or part of No. SCI-AK) that enters from the top of the injector. The advantage of the modified system is that injector contamination can be seen and the used insert can be easily cleaned. A 7 cm needle syringe is required for the modified injector.

(b) *Gas chromatograph.*—Perkin-Elmer Sigma 3B, with 2 m × 4 mm id pyrex column packed with 1% SE-30 on 100–120 mesh Gas-Chrom Q. Operating conditions: temperature (°C)—injector 300, column 250, detector 300. The relative retention times (min) for the major sterols in this study were: cholesterol 0.60–0.62, brassicasterol 0.68–0.70, campesterol 0.80–0.82, β-sitosterol 1.00. Carrier nitrogen flow was ca 40 mL/min with minor flow rate adjustments to elute cholesterol in ca 15 min.

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(c) *Methylene chloride, methanol, pyridine, and n-hexane.*—Distilled in glass (Burdick & Jackson Laboratories, Muskegon, MI).

Preparation of Standards

Cholesterol, campesterol, and β-sitosterol standards were derivatized as previously described (9). Briefly, aliquots of standard dissolved in hexane were evaporated to dryness and refluxed with 4 mL butyric anhydride–pyridine (2 + 1) for 30 min. Solutions were then evaporated to near dryness and diluted to volume with hexane.

Preparation of Samples

Rapeseed, castor, and sunflower oils were used directly without further purification or extraction to prepare the butyrate derivatives of the sterols as previously described (9).

For peppercorns, 25 g pink berries was homogenized in a Model 45 Macro-Virtis homogenizer equipped with a 250 mL bowl containing ca 175 mL methylene chloride–methanol (2 + 1). The sample was homogenized ca 30 min until the berry fragments appeared finely divided. The homogenate was extracted by the procedure of Chen et al. (18). The total sterols were isolated from the extract by using preparative TLC plates of 0.5 mm Baker silica gel 7GF, using the solvent system and developing conditions of Malins and Mangold (19), which were further described by Mangold (20). The sterol band of the plate was located using an iodine vapor chamber, boundaries were scored, and the area was scraped into a 70 mm funnel containing an S&S 588 (or equivalent) filter paper. The sterols were extracted with five 10 mL portions of methylene chloride into a 100 mL beaker and evaporated to near dryness under a nitrogen stream on a steam bath. The sterols were then transferred to a 50 mL Erlenmeyer flask and diluted to volume with methylene chloride. An aliquot of this solution was derivatized as previously described. Olive oil, soybean oil, tobacco, and cocoa bean were extracted by the method of Chen et al. (18), and the sterol fraction was isolated using preparative TLC and derivatized as described above.

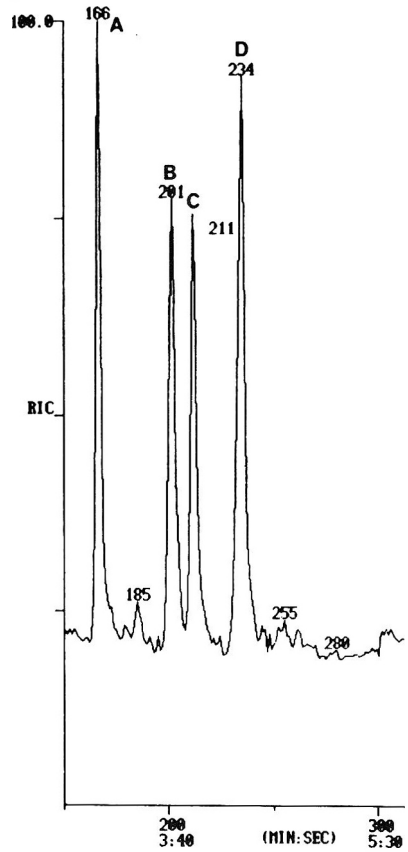

Figure 1. Total ion current (100–500 u) chromatogram (EI, 70 eV) of sterol butyrates. A, cholesterol; B, campesterol; C, stigmasterol; and D, β-sitosterol.

Results and Discussion

EI MS of Sterol Butyrates

Figure 1 illustrates the chromatography as the total ion current that was obtained using capillary column GC/MS for cholesterol, campesterol, stigmasterol, and β-sitosterol standards as their butyryl esters. Brassicasterol butyrate elutes between cholesterol and campesterol butyrate. Figures 2A–E present the mass spectra of these 5 common sterol butyrates. As a reference point of discussion, the mass spectrum of cholesterol butyrate (Figure 2A) is considered in detail. The highest mass ion observed is found at m/z 368 and may be represented as $(M\text{-}ROH)^{+\cdot}$ ions, where R = $CH_3CH_2CH_2C(O)$. This behavior is in contrast to TMS ether derivatives of Δ^5 sterols, which often show appreciable molecular ions as well as the $(M\text{-}TMSOH)^{+\cdot}$ ion (17). Acetate and trifluoroacetate derivatives also yield relatively abundant $(M\text{-}R^1OH)^{+\cdot}$ ions, where R^1 represents the acetyl or trifluoroacetyl group (17). However, the specificity of the mass spectrum of cholesterol butyrate remains high because of the characteristic fragment ions observed. Fragment ions above m/z 200 include m/z 353, 260, 255, 247, and 213, corresponding to ions represented as $(M\text{-}ROH\text{-}15)^+$, $(M\text{-}ROH\text{-}108)^{+\cdot}$, $(M\text{-}ROH\text{-}SC)^+$, $(M\text{-}ROH\text{-}121)^+$, and $(M\text{-}ROH\text{-}SC\text{-}42)^+$, where SC = side chain group (113 u for cholesterol butyrate) (17). This fragmentation pattern most closely resembles that found in the spectra of sterol acetates (17), and is repeated in the spectra of the other Δ^5 sterol butyrates of campesterol and β-sitosterol (Figures 2B and D) with appropriate changes in mass due to different side chains. The spectra of diunsaturated sterol butyrates of brassicasterol and stigmasterol (Figures 2E and C) afford similar fragmentation but differ in the greater relative abundance of the ions at m/z 255 $(M\text{-}ROH\text{-}SC)^+$ and 253 $(M\text{-}ROH\text{-}SC\text{-}2H)^+$ as well as in the presence of m/z 282. The latter ion may represent loss of most of the side chain with possible cleavage between C_{20} and C_{22} accompanied by hydrogen rearrangement. The $\Delta^{5,22}$ sterol butyrates show a relatively greater abundance for the ion represented as $(M\text{-}ROH\text{-}43)^+$ as well (17). Since the fragment ions in the spectra of sterol butyrates all involve loss of ROH in addition to other units, the overall pattern above m/z 200 is simpler than that found in spectra of free sterols and other sterol derivatives, which show additional fragment ions not involving loss of the R^1OH unit (i.e., water, acetate, and TMSOH).

The fragment ion patterns below m/z 200 are generally indicative of sterols. They consist of ion clusters centered at m/z 107, 119, 133, 145, 159, 173, 185, and 199. Fragmentations leading to these ions are probably complex. Ions representative of the butyryl group such as m/z 71 are present at about the same relative abundance as ions in the limited mass range usually scanned (100–500 u). In contrast, positive ion chemical ionization (methane) spectra are dominated by m/z 89 representative of protonated butyric acid and weak ions indicative of the sterol structure (unpublished observations). Thus, chemical ionization MS does not appear to be as useful as EI MS for identification of sterol butyrates.

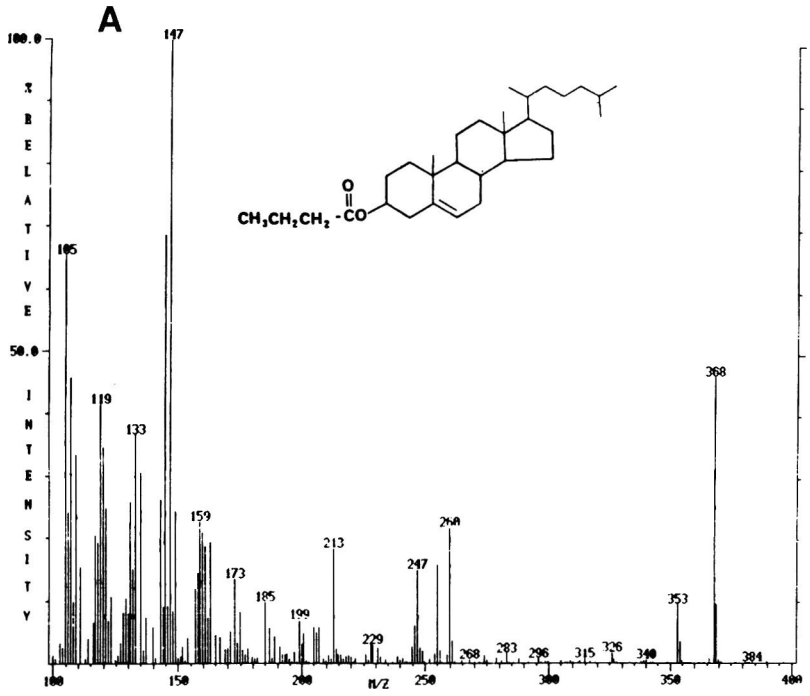

Figure 2A. EI mass spectrum (GC introduction, 70 eV) of cholesterol butyrate.

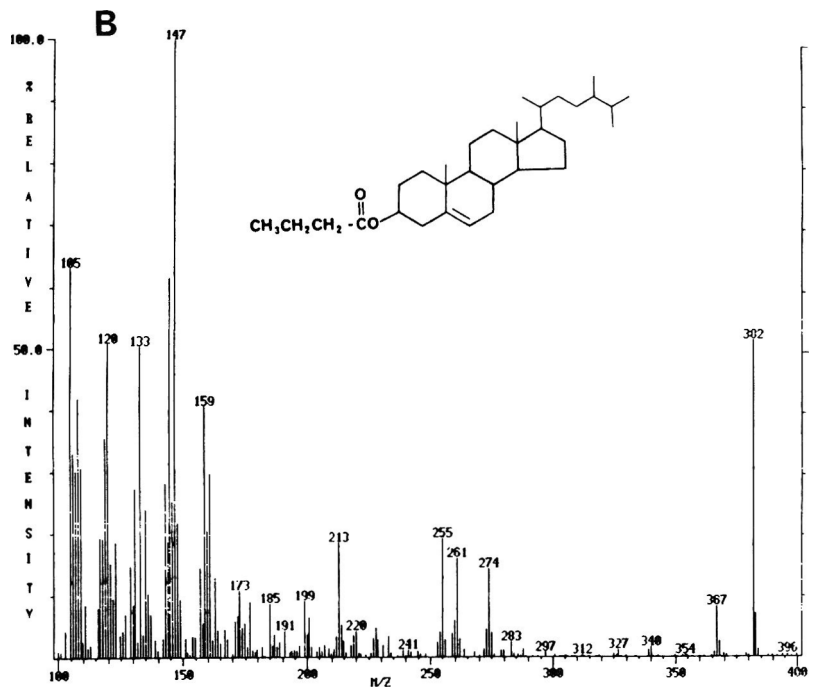

Figure 2B. EI mass spectrum (GC introduction, 70 eV) of campesterol butyrate.

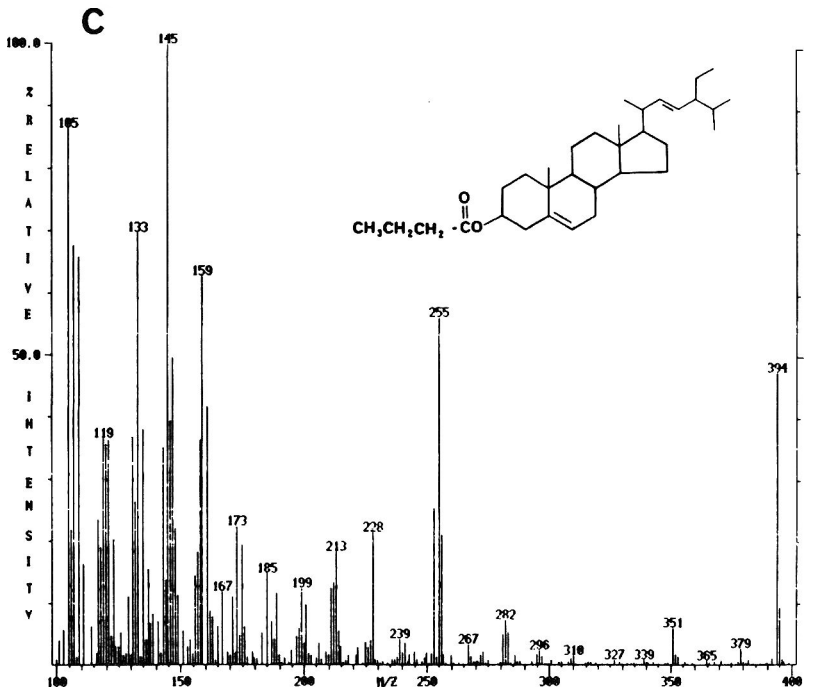

Figure 2C. EI mass spectrum (GC introduction, 70 eV) of stigmasterol butyrate.

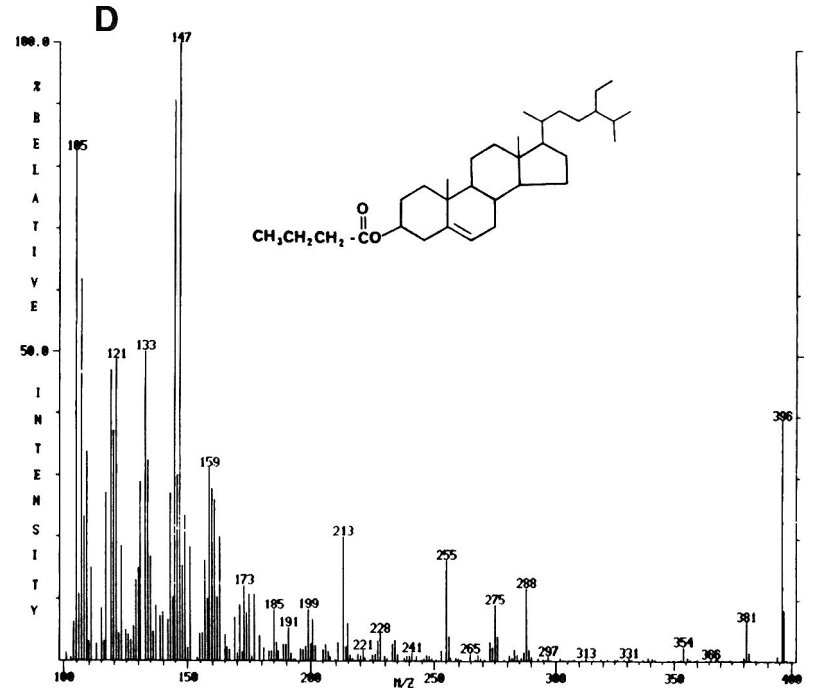

Figure 2D. EI mass spectrum (GC introduction, 70 eV) of β-sitosterol butyrate.

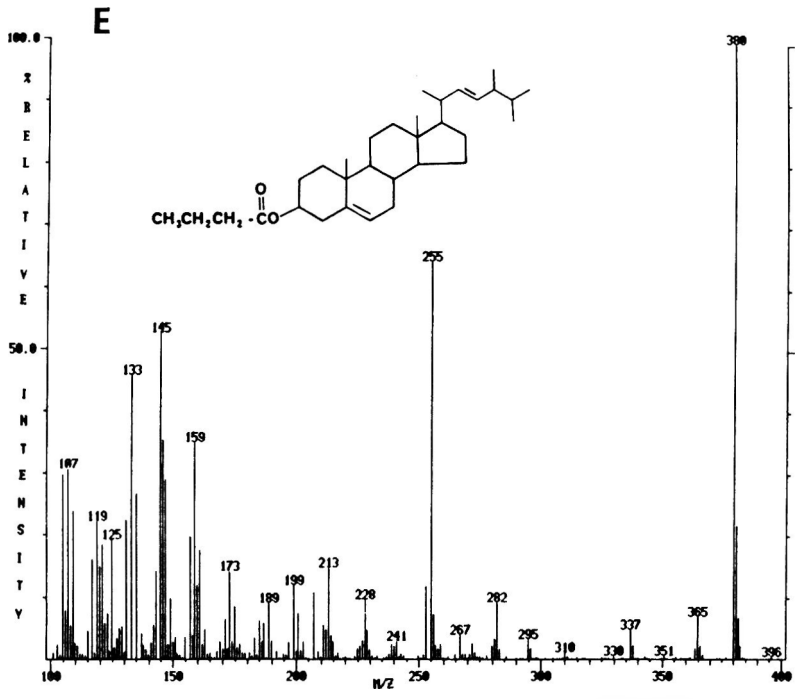

Figure 2E. EI mass spectrum (GC introduction, 70 eV) of brassicasterol butyrate.

EI MS of Virgin Olive Oil

Figure 3A illustrates the total ion chromatogram obtained from a sample of virgin olive oil. Typically, 3 components are found with retention times (RTs) longer than β-sitosterol. We have tentatively identified the components at RT = 12:18 min:sec as cycloartanol (m/z 408 = M-ROH)$^{+\cdot}$) and at RT = 12:37 min:sec as 24-methylenecycloartanol (m/z 422 = (M-ROH)$^{+\cdot}$) Figures 3B and C). The spectrum of the latter compound reveals characteristic fragment ions at m/z 407, 379, 353, 339, 325, and 300 corresponding to (M-ROH-15)$^+$, (M-ROH-43)$^+$, (M-ROH-69)$^+$, (M-ROH-83)$^+$, (M-ROH-97)$^+$, and (M-ROH-122)$^{+\cdot}$. This tentative identification was further corroborated by obtaining spectra of the free sterols in the underivatized sample and comparing them with reference spectra (21).

The third component observed at RT 12:47 min:sec appears to be steroidal in nature, but ions afforded by the butyryl ester above m/z 200 do not result in a fragmentation pattern readily related to one of those already described. Ions were observed at m/z 398, 355, 326, 310, 295, 267, 241, and 227. High resolution GC/MS mass measurements were consistent with elemental compositions of $C_{27}H_{42}O_2$ for m/z 398, $C_{24}H_{35}O_2$ for m/z 355, and $C_{23}H_{34}$ for m/z 310. Examination of the mass spectra afforded by the underivatized sample indicated that an additional minor component present was likely a 4,4-dimethylsterol such as cyclobranol or the Δ^{25} isomer, but no component was observed in the free sterols that could be related to the butyryl ester of the unknown. The lack of obvious correlation of the spectrum of the unknown in the derivatized sample with known compounds prevents identification.

Other Sterol Butyrates

We also report the spectra of the butyryl esters of fucosterol, lanosterol, and stigmastanol (Figures 4A–C) for comparison with results already discussed. The spectrum of fucosterol butyrate (Figure 4A) resembles those of the other sterol butyrates, including stigmasterol butyrate, with the highest mass ion corresponding to (M-ROH)$^{+\cdot}$ at m/z 394. Ions at m/z 281, 255, 253, and 213 are also common to the spectrum of stigmasterol butyrate. The most notable feature of the spectrum of fucosterol butyrate is the base peak at m/z 296 corresponding to (M-ROH-88)$^+$ resulting from cleavage between C_{22} and C_{23} with hydrogen transfer to the neutral fragment. Ions at m/z 314 and 296 are observed in the spectrum of the free sterol corresponding to (M-88)$^{+\cdot}$ and (M-H_2O-88)$^{+\cdot}$.

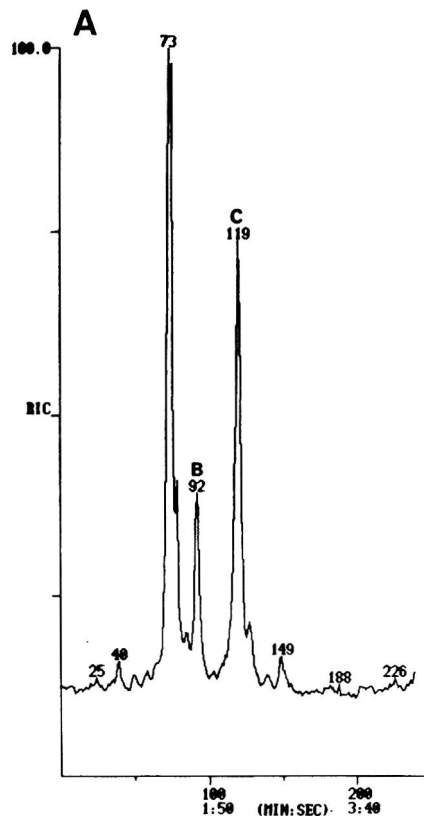

Figure 3A. Total ion current (100–500 u) chromatogram (EI, 70 eV) of sterol butyrates from virgin olive oil.

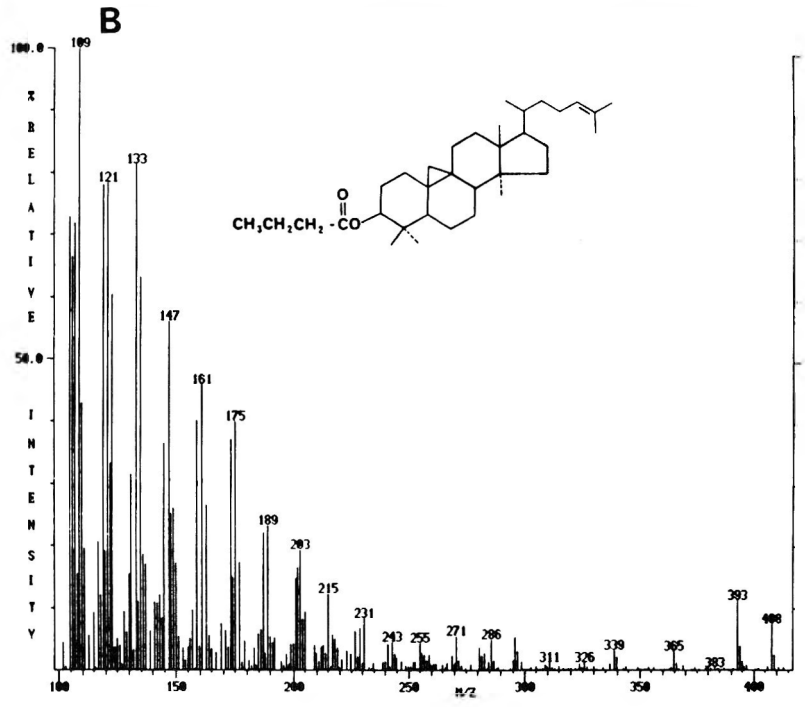

Figure 3B. EI mass spectrum of components at RT 12:18.

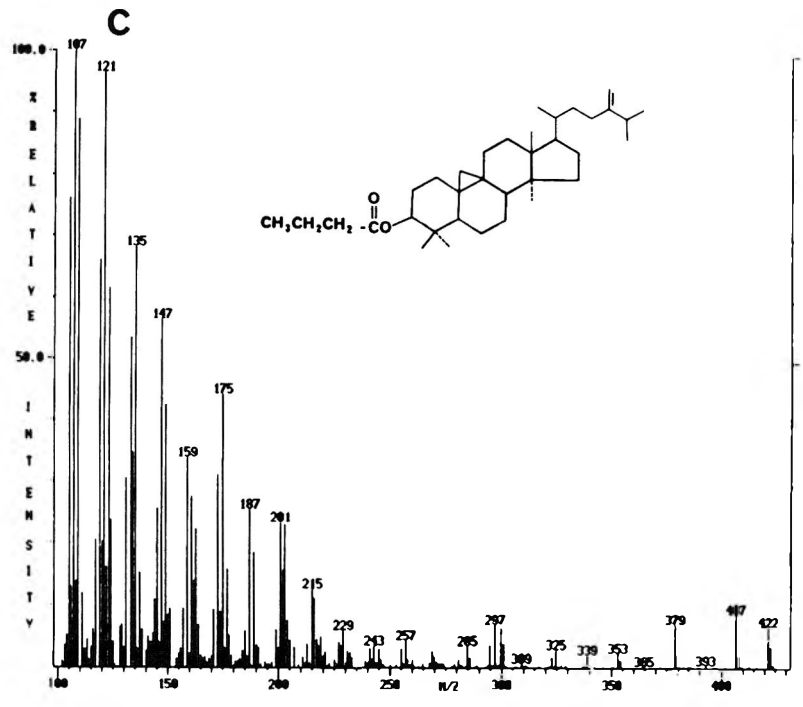

Figure 3C. EI mass spectrum of components at RT 12:37.