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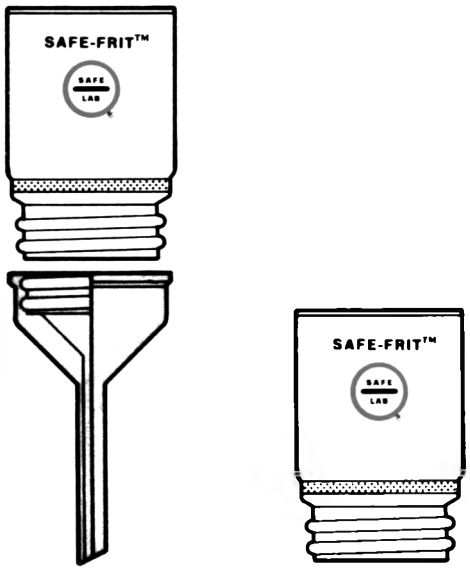
ASSOCIATION OF OFFICIAL ANALYTICAL CHEMISTS

JOURNAL

JANUARY/FEBRUARY 1987
VOL. 70, NO. 1

JANCA 70(1) 1–180 (1987)
ISSN 0004-5756

SAFE-FRIT® FUNNELS



SAFE-LAB, INC. introduces a **NEW** generation of fritted glass funnels. The SAFE-FRIT™ funnel features a threaded removable unbreakable polypropylene stem just below the fritted glass disc. This unique design eliminates the danger of broken glass funnel tips and allows easy and safe cleaning from both sides. The polypropylene stem has excellent chemical resistance and the threads will not allow leakage even at high vacuum. The high quality borosilicate glass disc is available in five porosity sizes.

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POROSITY	PORE SIZE	APPLICATION
Coarse	40 - 60 µ	For mercury filtration and very coarse materials.
Medium	10 - 15 µ	For filtering organic and most inorganic crystalline precipitates.
Fine	4 - 5.5 µ	For filtering fine precipitates such as barium sulfate and cuprous oxide.
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Ultra Fine	0.9 - 1.4 µ	For bacterial filtration.

SAFE-FRIT™ FUNNEL, COARSE POROSITY

CATALOG NUMBER	VOLUME in ml	DISC DIA, mm	STEM DIA, mm	PRICE EACH
F-3000-C	30	30	10	$ 21.60
F-3002-C	125	50	10	49.00
F-3004-C	350	65	26	74.20
F-3006-C	500	90	26	96.80
F-3008-C	1000	100	26	126.10

SAFE-FRIT™ FUNNEL, MEDIUM POROSITY

CATALOG NUMBER	VOLUME in ml	DISC DIA, mm	STEM DIA, mm	PRICE EACH
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F-3002-M	125	50	10	49.00
F-3004-M	350	65	26	74.20
F-3006-M	500	90	26	96.80
F-3008-M	1000	100	26	126.10

SAFE-FRIT™ FUNNEL, FINE POROSITY

CATALOG NUMBER	VOLUME in ml	DISC DIA, mm	STEM DIA, mm	PRICE EACH
F-3000-F	30	30	10	$ 23.80
F-3002-F	125	50	10	52.90
F-3004-F	350	65	26	79.80
F-3006-F	500	90	26	109.00
F-3008-F	1000	100	26	149.19

SAFE-FRIT™ FUNNEL, VERY FINE POROSITY

CATALOG NUMBER	VOLUME in ml	DISC DIA, mm	STEM DIA, mm	PRICE EACH
F-3000-VF	30	30	10	$ 26.75
F-3002-VF	125	50	10	60.59
F-3004-VF	350	65	26	83.88
F-3006-VF	500	90	26	125.86
F-3008-VF	1000	100	26	181.18

SAFE-FRIT™ FUNNEL, ULTRA FINE POROSITY

CATALOG NUMBER	VOLUME in ml	DISC DIA, mm	STEM DIA, mm	PRICE EACH
F-3000-UF	30	30	10	$ 32.86
F-3002-UF	125	50	10	75.60
F-3004-UF	350	65	26	97.83
F-3006-UF	500	90	26	149.10
F-3008-UF	1000	100	26	236.10

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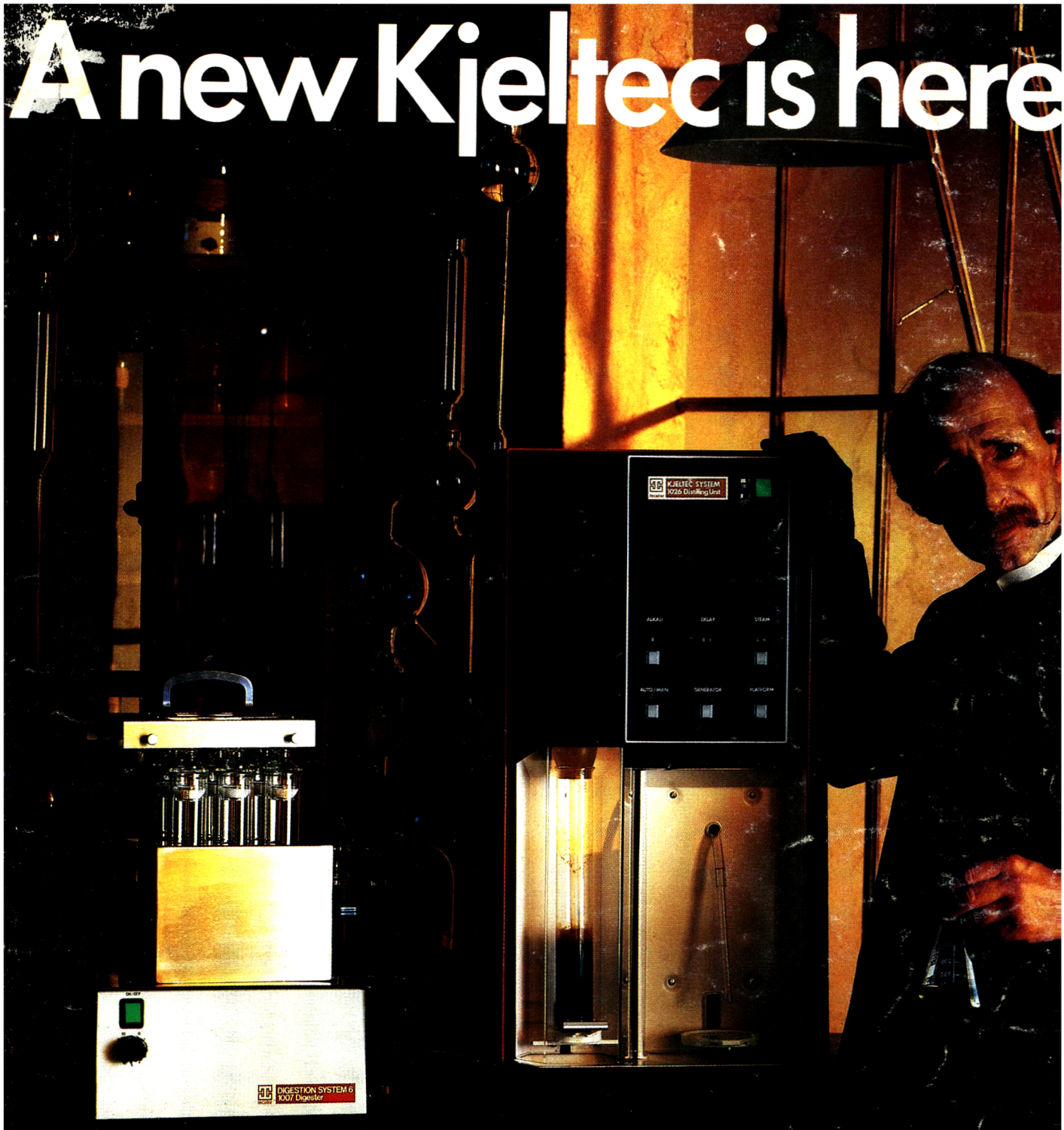

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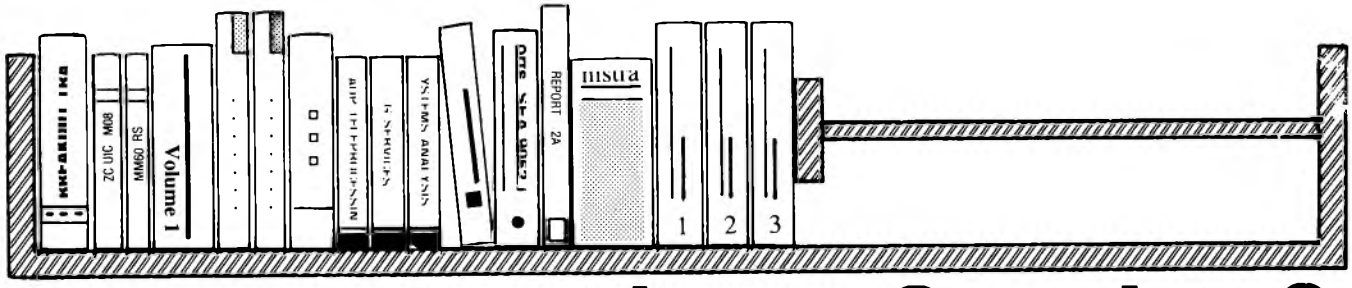

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A handbook for initiating or improving a laboratory quality assurance program.

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A do-it-yourself manual for statistical analysis of interlaboratory collaborative tests.

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A basic reference for evaluating collaborative studies and a natural extension to the *Statistical Manual of the AOAC*.

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Chromatographic and spectroscopic techniques with analyses for specific cosmetics.

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The *Journal of the Association of Official Analytical Chemists* (ISSN 0004-5756) is published bimonthly by AOAC, 1111 N 19th St, Suite 210, Arlington, VA 22209. Each volume (one calendar year) will contain about 1200 pages. The scope of the *Journal* encompasses the development and validation of analytical procedures pertaining to the physical and biological sciences related to foods, drugs, agriculture, and the environment. Emphasis is on research and development of precise, accurate, and sensitive methods for the analysis of foods, food additives and supplements, contaminants, cosmetics, drugs, toxins, hazardous substances, pesticides, feeds, fertilizers, and the environment.

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ASSOCIATION OF OFFICIAL ANALYTICAL CHEMISTS

JOURNAL

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VOL. 70, NO. 1
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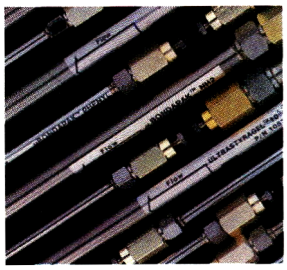

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Circle No. 1 on reader service card.

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Four new enzymatic test kits to detect formic acid, nitrate, succinic acid, and lactose/D-glucose have been introduced by Boehringer Mannheim Biochemicals. These kits are sensitive, rapid, easy to use, accurate, and highly specific. Each comes with all reagents, protocols for sample preparation, and an assay procedure. Boehringer Mannheim Biochemicals.

Circle No. 2 on reader service card.

Fourier Transform Infrared Spectroscopy

RFX-75 is the name given Analect Instruments' new modular high resolution FTIR spectrometer. The instrument's sampling capabilities, data system performance, and software can be continuously upgraded and expanded by the user. It is built around a patented multiple-task optical system, multiprocessor data system, and software that can be run in conjunction with any third-party software written in MS/DOS. The RFX-75 provides 0.12 cm resolution and 86 Mbyte hard disk storage, with options including a 400 Mbyte optical disk storage unit. Analect Instruments.

Circle No. 3 on reader service card.

External vibrations and ambient temperature changes will not affect the Michelson 100, enabling the use of IR spectroscopy in both laboratory and industrial environments. The interferometer of the Michelson 100 is free of mechanical or air bearings, is insensitive to optical misalignments, and is housed in a sealed enclosure, eliminating the need for purging the interferometer. Bomem, Inc.

Circle No. 4 on reader service card.

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Circle No. 5 on reader service card.

Infrared Microscope Series

Three high performance infrared microscope configurations for use with all commercially available FTIR spectrometers are being introduced by Spectra-Tech, Inc. They combine features of research grade visible light microscopy and infrared microscopy for FTIR sampling and are intended for use in such areas as forensics, analytical chemistry, manufacturing, and electronics. Spectra-Tech, Inc.

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The new Model 3A30 amino acid analyzer, a compact, self-contained unit with a built-in autosampler, holds up to 100 samples and can be used to measure all amino acids in physiological fluids and hydrolysates as well as sugars and polyamines. Equipped with a microprocessor programmer with video display, it is suitable for research, quality control, or diagnostic applications in clinical, biochemical, agricultural, or food and beverage laboratories. Carlo Erba Strumentazione.

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monitor. COMPress.
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C.R.I.'s line of laser power stabilizers, LS-100 and LS-200, remove high-speed noise and low-speed drift to allow researchers to attain increased signal-to-noise in fluorescence and Raman spectroscopy experiments and in sample transmission, scattering, and turbidity measurements. Cambridge Research & Instrumentation, Inc.
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Supercritical Fluid Chromatography Systems

Brownlee Labs, Inc., has published a product bulletin providing a complete description of and price information on its System One supercritical fluid chromatography unit. The bulletin also gives suggestions on when to use SFC and the suitability of different sample types for SFC. Brownlee Labs, Inc.
Circle No. 18 on reader service card.

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Circle No. 23 on reader service card.

Total Organic Carbon Analyzer

Dohrmann offers an 8-page brochure describing the new DC-180 TOC analyzer. The instrument uses UV-persulfate oxidation and can directly measure total carbon, inorganic carbon, and purgeable and nonpurgeable organic carbon in water. Dohrmann Division, Xertex Corp.
Circle No. 24 on reader service card.

Confirmational Analysis GC System

An instrument system for the confirmational analysis of chlorinated hydrocarbons using a dual-oven gas chromatograph is now available from IBM Instruments, Inc. It is designed specifically for use by environmental, pharmaceutical, food, chemical, and other industrial laboratories where regulatory methods require confirmation of component identification. IBM Instruments, Inc.
Circle No. 25 on reader service card.

Helium Afterglow Discharge Detector

The CETAC HeAD-100 is a helium afterglow discharge detector designed for use with a gas chromatograph. It is effective in detecting environmental pollutants such as organometallic forms of arsenic, mercury, and lead; it can also differentiate between sulfur- and phosphorus-containing compounds present in pesticides and among halide-containing organic compounds. After a sample elutes from the gas chromatograph, it is injected into the HeAD. The sample particles collide with highly energized helium atoms and molecules; the collision dissociates and energizes the sample, and as the sample atoms lose energy, they emit characteristic optical spectra. Ames Laboratory.
Circle No. 26 on reader service card.

Disposable Micropartition System

An ultrafiltration device for preparing samples in food research or food quality control laboratories is offered by Amicon division of W. R. Grace & Co. The Centrifree separates interfering, particulate, and macromolecular components from food samples before analysis for low molecular weight species. Amicon.
Circle No. 27 on reader service card.

Automatic Preparative Gas Chromatograph

The PSGC-10/40, an automatic preparative gas chromatograph, has been introduced by the Varex Corp. for separation, isolation, and purification of pharmaceuticals, flavors, fragrances, fine chemicals, natural products, and other chemicals. It is suited for resolution of azeotropes, structural isomers, and closely boiling materials that are difficult to purify by other means. Varex Corp.
Circle No. 28 on reader service card.

Hazardous Chemicals Information Service

Emergency handling information regarding 1016 hazardous chemicals is available from a database on the Chemical Information System. Chemical Hazard Response Information System (CHRIS), developed by the U.S. Coast Guard, is designed to aid spill-response personnel in emergency situations and can also be used in the development of procedures for avoidance of emergencies. Chemical Information Systems, Inc.
Circle No. 29 on reader service card.

Siliconized Glassware

I-Chem siliconized glass sample containers protect against leaching of trace metals into solution, reduce adsorption of polar compounds, proteins and trace metals, and protect samples against the possible effects of -OH sites often present on glass. I-Chem Research.
Circle No. 30 on reader service card.

Audiovisual Training for Gas Chromatography

Savant offers 4 audiovisual training packages in gas chromatography, the newest being "Capillary Columns in Gas Chromatography," by Harold McNair of Virginia Polytechnic Institute and State University in Blacksburg, VA. All programs are available in slide/tape or video formats and run between 20 and 40 min. Savant.
Circle No. 31 on reader service card.

FOR YOUR INFORMATION

Meetings

February 14–18, 1987: 153rd national meeting, American Association for the Advancement of Science, Hyatt Regency Chicago, Chicago, IL. Contact: Joan Wrather, AAAS, 1333 H St, NW, Washington, DC 20005, telephone 202/326-6440.

March 3–6, 1987: II World Congress of Food Technology, Barcelona, Spain. Contact: Inter-Congres, Gran Via, 646, 4.°, 4.ª, 08007 Barcelona, Spain, telephone (93) 301 25 77.

April 12–15, 1987: Twelfth National Nutrient Databank Conference, Westin Galleria Houston Hotel, Houston, TX. Contact: Twelfth National Nutrient Databank Conference, The University of Texas System Cancer Center, M. D. Anderson Hospital and Tumor Institute, Department of Cancer Prevention and Control-HMB 189, 6723 Bertner Ave, Houston, TX 77030, telephone 713/792-7760.

April 27–30, 1987: 12th AOAC Spring Training Workshop and Exhibition, Skyline Ottawa Hotel, Ottawa, Ontario, Canada. Contact: Graham MacEachern, Agriculture Canada, Ottawa, Ontario K1A 0L2, Canada, telephone 613/994-1991; James Lawrence, Health and Welfare Canada, Tunney's Pasture, Ottawa, Ontario K1A 0L2, Canada, telephone 613/990-8459.

May 12–14, 1987: HAZTECH Canada, hazardous materials and waste management exhibition and conference, Toronto International Centre, Mississauga, Ontario, Canada. Contact: Beverly Gibson, Exhibition Management Co., 6143 S Willow Dr, Suite 100, Englewood, CO 80111, telephone 303/850-9119.

May 17–20, 1987: Annual Meeting of the Canadian Institute of Food Science and Technology, Hamilton Convention Centre, Hamilton, Ontario, Canada. Contact: Anne Goldman, 111 Churchill Rd S, Acton, Ontario L7J 2J5, Canada, telephone 519/853-3021.

May 19–21, 1987: Eighth International Symposium on Capillary Chromatography, Palazzo dei Congressi, Riva Del Garda, Italy. Contact: P. Sandra, Research Institute for Chromatography, PO Box 91, B-8610 Wevelgem, Belgium.

May 20–22, 1987: Analytical Biochemistry in Biotechnology: A Workshop, Sheraton Inner Harbor Hotel, Baltimore, MD. Contact: Cindi Coffman, Analytical Biochemistry in Biotechnology, 49 Sobey St, Charleston, MA 02129, telephone 617/241-8468.

June 15–16, 1987: AOAC Midwest Regional Section Meeting, Fargo, ND. Contact: Howard Casper, Veterinary Diagnostic Laboratory, North Dakota State University, Fargo, ND 58102, telephone 701/237-7529.

June 22–24, 1987: AOAC Northeast Regional Section Meeting, Wells College, Aurora, NY. Contact: George Wilkens, Agway Inc., 777 Warren Rd, Ithaca, NY 14850, telephone 607/257-2345.

June 25–26, 1987: AOAC Northwest Regional Section Meeting, Evergreen State College, Olympia, WA. Contact: H. Michael Wehr, Oregon Department of Agriculture, Laboratory Services Division, 635 Capitol St, NE, Salem, OR 97310, telephone 503/378-3793.

August 2–6, 1987: 29th Rocky Mountain Conference, sponsored by Rocky Mountain Section Society for Applied Spectroscopy and Rocky Mountain Chromatography Discussion Group, Radisson Hotel-Denver, Denver, CO. Contact: Michael Reddy, U.S. Geological Survey, 5293 Ward Rd, Arvada, CO 80002, telephone 303/236–3617.

September 14–17, 1987: 101st AOAC Annual International Meeting and Exhibition, The Cathedral Hill Hotel, San Francisco, CA. Contact: Margaret Ridgell, AOAC, 1111 N 19th St, Suite 210, Arlington, VA 22209, telephone 703/522-3032.

September 17–18, 1987: 2nd Seminar of the European Organization for Quality Control, Section for Quality Control in the Food Industry, Zurich, Switzerland. Contact: Secretariat, EOQC-Section for QC in the Food Industry, PO Box 2613, CH-3001 Berne, Switzerland, telephone 21 61 11, telex 912 110.

September 27–October 2, 1987: World Conference on Biotechnology for the Fats and Oils Industry, organized by American Oil Chemists' Society and Deutsche Gesselschaft für Fettwissenschaft, Hamburg, West Germany. Contact: Joan Dixon, AOCS Meetings Manager, 508 S Sixth St, Champaign, IL 61820, telephone 217/359-2344, telex 404472 OIL CHEM CHN.

October 20–21, 1987: AOAC Europe, Paris, France. Contact: Margreet Lauwaars, PO Box 153, 6720 AD Bennekom, The Netherlands, telephone 31-8389-1-8725.

August 29–September 1, 1988: 102nd AOAC Annual International Meeting and Exhibition, The Breakers, Palm Beach, FL. Contact: Margaret Ridgell, AOAC, 1111 N 19th St, Suite 210, Arlington, VA 22209, telephone 703/522-3032.

September 25–28, 1989: 103rd AOAC Annual International Meeting and Exhibition, The Clarion Hotel, St. Louis, MO. Contact: Margaret Ridgell, AOAC, 1111 N 19th St, Suite 210, Arlington, VA 22209, telephone 703/522-3032.

Short Courses

J. T. Baker Chemical Co. plans a series of one-day programs entitled "Solid Phase Extraction Course and Laboratory Workshop," which will focus on a practical and applications-oriented approach to solid phase extraction sample preparation. Courses will be conducted in 1987 in Orange County and San Francisco, CA, in February; in Research Triangle Park, NC, in March; in St. Louis, MO, and Minneapolis/St. Paul, MN, in April; in Pittsburgh, PA, and Cincinnati, OH, in May; and in Houston and Dallas, TX, in June. Cost for the first attendee is $125.00, for additional attendees, $85.00. Contact: Course Coordinator, J. T. Baker Chemical Co., 222 Red School Lane, Phillipsburg, NJ 08865, telephone 800/582-2537 (New Jersey 201/859-2151).

"Hazardous Waste Management for Small Generators: Preparation for Disposal of Laboratory Chemicals" is the title of a one-day course offered by the University of Southern California Institute of Safety and Systems Management. The program is designed to assist health and safety professionals to comply with state and federal regulations that deal with the special problems evolving from the handling and disposal of hazardous laboratory wastes. Credit is given for attendance. Dates are March 13, 1987, or August 31, 1987; cost is $150.00. Contact: University of Southern California, University Gardens, 3500 S Figueroa St, Suite 105, Los Angeles, CA 90007, telephone 213/743-6523.

AOAC plans 3 Quality Assurance Short Courses in 1987. These 2-day courses teach how to plan, design, and manage a laboratory quality assurance program. Courses are scheduled for April 7–8 and July 28–29 at the Westpark Hotel, Arlington, VA, and for September 12–13 at the Cathedral Hill Hotel, San Francisco, CA. Cost to AOAC members is $475.00, to nonmembers is $525.00. Contact: Margaret Ridgell,

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Administrative Manager
AOAC, 1111 N. 19th Street, Suite 210
Arlington, VA 22209, (703) 522-3032

AOAC, 1111 N 19th St, Suite 210, Arlington, VA 22209, telephone 703/522-3032.

Collaborative Study of the Year Award—1986

In recognition of his collaborative study "Rapid LC Determination of Aflatoxin M_1 and M_2 in Artificially Contaminated Fluid Milk," Robert D. Stubblefield received the Collaborative Study of the Year Award for 1986 at the 100th AOAC Annual International Meeting and Exhibition, Scottsdale, AZ, Sept. 15–18, 1986.

This new award was created when the former Associate Referee of the Year award was replaced. Collaborative Study of the Year Award is intended to recognize the individual Associate Referee responsible for the collaborative study deemed best for the year on the basis of scientific innovation and soundness of design, implementation, and reporting.

Method Committee Associate Referee Awards for 1986

When the Official Methods Board replaced the Associate Referee of the Year award, they created, in addition to Collaborative Study of the Year, the Method Committee Associate Referee Awards to recognize the best Associate Referee in each committee for a given year. Named in 1986 were Stephen C. Slahck, Committee on Pesticides and Disinfectants; Susan Ting, Committee on Drugs; Robert D. Stubblefield, Committee on Foods I; Leon Prosky, Committee on Foods II; Susan C. Hight, Committee on Residues; Phyllis Entis, Committee on Microbiology; Michael Carlson, Committee on Feeds, Fertilizers and Related Areas; and Paul H. Friedman, Committee on Hazardous Substances in Waste and the Environment.

Announcement of New Journal

The Chemometrics Society has announced a new journal, *Journal of Chemometrics*, a quarterly whose first issue appeared in autumn 1986. According to the announcement of the journal, "the specific aim . . . is to publish papers on both fundamental and applied aspects of chemometrics, and to provide a forum for the exchange of information on meetings, etc., for the growing international chemometrics research community . . . As chemometrics is a science that bridges chemistry and applied mathematics and statistics, [the editors] intend to create the journal as an invaluable aid to researchers and educators in all three areas. The journal will also provide a facility for the exchange of software and the publication of computer programmes." Editor-in-chief of the new journal is Bruce R. Kowalski of the University of Washington, Seattle, WA. Authors should submit manuscripts to the nearest of the 2 associate editors: Steven D. Brown, Department of Chemistry, University of Delaware, Newark, DE 19716; Bernard G. M. Vandeginste, Department of Analytical Chemistry, Faculty of Science, Catholic University of Nijmegen, Nijmegen, The Netherlands. For subscription information, contact John Wiley & Sons Ltd, Baffins Lane, Chichester, West Sussex PO19 1UD, England.

New Private Sustaining Members

AOAC welcomes the following new private sustaining members: CEM

Corp., Indian Trail, NC; Wallace Laboratories, Cranbury, NJ; Laboratorio di Chimica Analitica Applicata S.N.C., Bologna, Italy; Fisher Scientific Co., Pittsburgh, PA; S. C. Johnson, Inc., Racine, WI; New Zealand Milk Products, Inc., Petaluma, CA; and Integrated Genetics, Framingham, MA

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 the prices indicated, from American National Standards Institute, Inc., 1430 Broadway, New York, NY 10018, telephone 212/354-3300.

ISO 6557/1-1986: Fruits, vegetables and derived products—Determination of ascorbic acid—Part I: Reference method. $14.00.

ISO 7466-1986: Fruit and vegetable products—Determination of 5-hydroxy-methylfurfural (5-MHF) content. $14.00.

ISO 7701-1986: Dried apples—Specification. $16.00.

ISO 7702-1986: Dried pears—Specification. $16.00.

ISO 7703-1986: Dried peaches—Specification. $16.00.

ISO 8086-1986: Dairy plant—Hygiene conditions—General guidance on inspection and sampling procedure. $16.00.

EPA Project Summary

The report entitled "Development of Standard Methods for the Collection and Analysis of Precipitation" describes the creation of an analytical methods manual to be used for acid deposition studies. The manual is intended to provide the scientific community with a set of standardized procedures for collecting and analyzing wet precipitation samples. Methodologies include flame atomic absorption spectrophotometry, ion selective electrode, automated colorimetry, ion chromatography, and titrimetric procedures. The adoption of standard test procedures will lead to greater comparability between laboratories reporting precipitation chemistry data and will improve the reliability of data interpretation efforts. The full report (Order No. PB 86-201 365/AS) can be obtained for $22.95 (subject to change) from National Technical Information Service, 5285 Port Royal Rd, Springfield, VA 22161, telephone 703/487-4650. For further information, contact: John D. Pfaff, Environmental Monitoring and Support Laboratory, U.S. Environmental Protection Agency, Cincinnati, OH 45268.

Interim Method

The following method has been accepted interim first action by the methods Committee on Microbiology and the Chairman of the Official Methods Board: Identification of Mammalian Feces by Coprostanol Thin Layer Chromatography, submitted by G. P. Hoskin (Food and Drug Administration, Division of Microbiology, Washington, DC). The method will be submitted for adoption as official first action at the 101st AOAC Annual International Meeting, Sept. 14–17, 1987, at San Francisco, CA. Copies of the method are available from the AOAC office.

Correction

J. Assoc. Off. Anal. Chem. (1985) **68,** 622–625, "Volatile Oil Analysis of Cassia Bark (Cinnamon): Investigation of Systematic Errors," by Way, p. 622, left column, Abstract, next to last sentence, change to read, ". . . suggesting there is a significant difference between the results reported by the 6 laboratories."

REGIONAL AOAC MEETINGS

June 15-16, 1987 — **Midwest Regional Section Meeting**
Fargo, North Dakota
Contact: Howard Casper, North Dakota State University
Veterinary Diagnostic Laboratory
Fargo, North Dakota, 58102
(701) 237-7529

June 22-24, 1987 — **Northeast Regional Section Meeting**
Wells College, Aurora, NY
Contact: George Willkens, Agway Inc.
777 Warren Road
Ithaca, NY 14850
(607) 257-2345

June 25-26, 1987 — **Northwest Regional Section Meeting**
Evergreen State College, Olympia, Washington
Contact: Mike Wehr, Oregon Department of Agriculture
Laboratory Services Division
635 Capitol Street, NE, Salem, Oregon 97310
(503) 378-3793

BOOKS IN BRIEF

Basic Tests for Pharmaceutical Substances. Published by the World Health Organization, WHO Publications Center USA, 49 Sheridan Ave, Albany, NY 12210, 1986. 204+ pp. Price: $17.00 (Sw fr 34.–). ISBN 92-4-154204-7.

This book presents simplified tests for verifying the identity and detecting gross degradation of 321 pharmaceutical substances. The book concludes with a list of required equipment and a description of the reagents, test solutions, and volumetric solutions mentioned in the basic tests. The procedures for the preparation of test solutions that require special attention are given in detail. English version available; Arabic, French, and Spanish versions in preparation.

Ultratrace Analysis of Pharmaceuticals and Other Compounds of Interest. Edited by Satinder Ahuja. Volume 85 of *Chemical Analysis: A Series of Monographs on Analytical Chemistry and Its Applications.* Published by John Wiley & Sons, Inc., One Wiley Dr, Somerset, NJ 08873, 1986. 384 pp. Price: $59.95. ISBN 0471-82673-1.

Analytical chemists and scientists who use ultratrace analyses in food, pharmaceutical, and environmental research should find this book helpful as a reference and research tool for in-depth studies of action, dispositions, and toxic properties of chemicals. It may also be an aid in determining the safety of food, water, and the environment and in determining the modes of action of drugs.

Analytical Applications of Lasers. Edited by Edward H. Piepmeier. Volume 87 of *Chemical Analysis: A Series of Monographs on Analytical Chemistry and Its Applications.* Published by John Wiley & Sons, Inc., One Wiley Dr, Somerset, NJ 08873, 1986. 703 pp. Price: $89.50. ISBN 0471-87023-4.

In this book, 22 experts offer an overview of contemporary laser technology in analytical chemistry. Among other topics, the book covers selected methods using various detection schemes, methods with improved spectral resolution, selected multiphoton and multiwavelength methods, and methods based on special characteristics of lasers.

Emulsions and Solubilization. By Kozo Shinoda and Stig Friberg. Published by John Wiley & Sons, Inc., One Wiley Dr, Somerset, NJ 08873, 1986. Price: $45.00. ISBN 0471-03646-3.

The authors discuss solution behavior of surfactants and the correlation of surfactants with emulsion type, stability, oil-water interfacial tension, and the solvent power of surfactant solutions. The book treats both the theoretical and practical aspects of the hydrophilic lipophilic balance concept in emulsions and microemulsions and offers a practical discussion of the specific properties of nonionic surfactants of the type polyethylene glycol alkyl (aryl) ethers.

Undergraduate Instrumental Analysis. 4th Edition. By James W. Robinson. Published by Marcel Dekker, Inc., 270 Madison Ave, New York, NY 10016, 1987. 664 pp. Price: $34.75 United States and Canada; $41.50 all other countries (subject to change without notice). ISBN 0-8247-7406-X.

Intended as a text for second-semester courses on quantitative analysis or more advanced courses on instrumental analysis, the book covers the more common instrumental methods used in analytical chemistry. It is written descriptively for a terminal course in analytical chemistry and is suited for both chemistry and nonchemistry majors.

Developments in Food Microbiology—2. Edited by R. K. Robinson. Published by Elsevier Applied Science Publishers, 52 Vanderbilt Ave, New York, NY 10017, 1986. Outside North America available from Elsevier Applied Science Publishers Ltd., Crown House, Linton Rd, Barking, Essex, England. 200 pp. Price: $49.50. ISBN 0-85334-432-9.

This volume presents topics ranging from the microbiology of table and fortified wines to some of the latest techniques in genetic engineering. Some other topics include the significance of the genera *Camplyobacter* and *Vibrio* as causes of food-borne disease and food poisoning from ingestion of naturally occurring plant toxins.

Microorganisms in Foods. 2. Sampling for microbiological analysis: Principles and specific applications. 2nd Edition. Sponsored by the International Commission of Microbiological Specifications for Foods of the International Union of Microbiological Societies. Published by University of Toronto Press, 63A St George St, Toronto, Ontario M5S 1A6, Canada, 1986. 293 pp. Price: $37.50. ISBN 0-8020-5693-8.

This edition of this book updates and reorganizes the first, which discussed the statistical aspects of sampling foods for microbiological analysis, presented the concept of 2- and 3-class attribute sampling plans, and suggested microbiological criteria for a range of food products and ingredients that move in international trade.

Seed Treatment. 2nd Edition. By K. A. Jeffs. Published by British Crop Protection Council Publications, 20 Bridport Rd, Thornton Heath, Surrey CR4 7QG, England, 1986. 332 pp. ISBN 0-948404-00-0.

This volume is intended to provide a comprehensive discussion of purposes of seed treatment, the methods used, and the technical and biological problems to be overcome for any given treatment to be effective. Primary consideration is given to the use of chemicals for the control of pests.

Organic Syntheses, Volume 64. Edited by Andrew S. Kende. Published by John Wiley & Sons, Inc., One Wiley Dr, Somerset, NJ 08873, 1986. 308 pp. Price: $27.50. ISBN 0471-84742-9.

This book is the next in a long-running series of volumes dedicated to the publication of experimental procedures which lead to compounds of wide interest or which illustrate important new developments in methodology.

Scientific Evidence in Criminal Cases. 3rd Edition. By Andre A. Moenssens, Fred E. Inbau, and James E. Starrs. Published by The Foundation Press, Inc., 170 Old Country Rd, Mineola, NY 11501-0509, 1986. 805 pp. Price: $32.95. ISBN 0-88277-281-3.

Significant portions of this edition have been rewritten or revised to increase comprehension, to add new material where warranted, and to eliminate superfluous material. For example, an

analysis of the statutory scheduling of controlled substances has been added, whereas the discussion of neutron activation analysis has been shortened consistent with the narrowing of the forensic application of NAA.

How to Manage a Scientific Laboratory. By Virginia White. Published by ISI Press, 3501 Market St, Philadelphia, PA 19104, 1986. 240 pp. Price: $39.95. ISBN 0-89495-065-7.

The author provides a complete set of guidelines concerning how to manage a research laboratory, offering practical advice on personnel selection, fiscal control, facilities and maintenance, how to run scientific meetings, publications, safety procedures, and other essential functions.

Handbook of Nonprescription Drugs. 8th Edition. Published by American Pharmaceutical Association, 2215 Constitution Ave, NW, Washington, DC 20037, 1986. 768 pp. Price: $70.00. ISBN 0-917330-54-4.

This 768 page reference provides detailed information on virtually every aspect of nonprescription medications, including practical information on their therapeutic groups and the conditions for which these products are used. More than 100 tables, completely updated for this edition, provide at-a-glance comparative information.

Reagent Chemicals. 7th Edition. Published by American Chemical Society, 1155 Sixteenth St, NW, Washington, DC 20036, 1986. 675 pp. Price: $89.95 United States and Canada; $107.95 export. ISBN 0-8412-0991-X.

Researchers will find definitions, procedures, standards, interpretation of requirements, precautions for tests, general directions, and more in this reference that provides test procedures and specifications and helps eliminate research errors caused by impure reagents.

Chromatographic Separations of Stereoisomers. By Rex W. Souter. Published by CRC Press, Inc., 2000 Corporate Blvd, NW, Boca Raton, FL 33431, 1986. 256 pp. Price: $99.00 United States; $144.00 outside United States. ISBN 0-8493-6127-3.

This volume organizes most of the significant currently available knowledge regarding the chromatographic separations of stereoisomers. Both diastereomers and the more difficult area of enantiomers are covered in depth with respect to gas chromatography, liquid chromatography, and classical chromatographic techniques.

Insecticides. Edited by D. H. Hutson and T. R. Roberts. Volume 5, *Progress in Pesticide Biochemistry.* Published by John Wiley & Sons, Inc., One Wiley Dr, Somerset, NJ 08873, 1986. 363 pp. Price: $87.95. ISBN 1-90758-8.

Pro-insecticides, those which convert into bioactivity by the metabolic activity of the target organism, are one topic of this volume. Others include resistance to pyrethroids, chitin synthesis inhibitors, and the natural chemicals which control insect behavior.

Compilation of ASTM Standard Definitions. 6th Edition. Sponsored by the ASTM Committee on Terminology. Published by American Society on Testing and Materials, 1916 Race St, Philadelphia, PA 19103, 1986. 907

pp., softbound. Price: $74.00 list; $59.20 member. ISBN 0-8031-0928-8.

The definitions in this reference work were written by people active in the field and reflect the sense in which that field uses the term in question. The purpose of the work is to eliminate the confusion and redundancy that exist in many ASTM standards.

Water Pollution by Fertilizers and Pesticides. Published by OECD Publications and Information Center, 1750 Pennsylvania Ave, NW, Washington, DC 20006-4582, 1986. 144 pp. Price: $12.00. ISBN 92-64-12856-5.

This report reflects the growing concern on the part of both agricultural and environmental experts that fertilizers, animal wastes, and pesticides are causing water pollution that exceeds acceptable levels in OECD countries. It looks at the potential consequences to health and impact on the water environment, and it proposes preventive measures and regulation schemes, research needs, changes in practice and management, and policy measures to reverse this trend.

PCB's and the Environment. Edited by John S. Waid. Published by CRC Press, Inc., 2000 Corporate Blvd, NW, Boca Raton, FL 33431, Vol. 1 and 2 due 1986, Vol. 3 due 1987. Vol. 1, 256 pp.; Vol. 2, 250 pp.; Vol. 3, 304 pp. Price (3-volume set): $415.00 United States; $475.00 outside United States. ISBN 0-8493-5929-5.

These volumes comprise in-depth reviews by an international panel of experts on the current state of knowledge of PCBs in the environment. Readers will find tabular data, descriptions of research methodologies, and detailed case studies involving a variety of ecological problems.

Gene Banks and the World's Food. By Donald L. Plucknett, Nigel J. H. Smith, J. T. Williams, and N. Murthi Anishetty. Published by Princeton University Press, 3175 Princeton Pike, Lawrenceville, NJ 08648, 1987. 248 pp. Price: $35.00. ISBN 0-691-08438-6.

Looking to the not-so-distant future, 4 experts urgently point out that we must not take our supply of seeds and other planting material for granted; the authors address a broad audience to show why gene banks are emerging as linchpins in the global effort to conserve as much of the gene pool of crop plants as possible. This book contributes to the crucial debate on how best to preserve some of society's most valuable raw material.

Evaluation of Pesticides in Ground Water. Edited by Willa Y. Garner, Richard C. Honeycutt, and Herbert N. Nigg. *ACS Symposium Series 315.* Published by American Chemical Society, 1155 Sixteenth St, NW, Washington, DC 20036, 1986. 586 pp. Price: $94.95 United States and Canada; $113.95 export.

This is the first book to address all factors that cause ground water contamination from pesticides. Although only pesticide contamination is examined, the principles can be applied to all substances that can reach ground water.

Agricultural Uses of Antibiotics. Edited by William A. Moats. *ACS Symposium Series 320.* Published by American Chemical Society, 1155 Sixteenth St, NW, Washington, DC 20036, 1986. 188 pp. Price: $39.95 United States and Canada; $47.94 export.

The major uses of antibiotics in agriculture are discussed here. Uses, risks and benefits, and regulatory aspects are all considered.

Personal Computers for Scientists: A Byte at a Time. By Glenn I. Ouchi. Published by American Chemical Society, 1155 Sixteenth St, NW, Washington, DC 20036, 1986. 300 pp. Price: paperbound—$22.95 United States and Canada; $27.95 export; hardbound—$34.95 United States and Canada; $41.95 export.

This volume walks you through the hardware and software capabilities of computers and tells you what PCs can do in the laboratory and which programs to use.

Instrumentation in Analytical Chemistry 1982-86. Edited by Stuart Borman. Published by American Chemical Society, 1155 Sixteenth St, NW, Washington, DC 20036, 1986. 326 pp. Price: paperbound—$24.95 United States and Canada; $29.95 export; hardbound—$39.95 United States and Canada; $47.95 export.

This comprehensive book looks at chemical microsensors, fiber optics, supercritical fluid chromatography, lab automation, hyphenated techniques, atomic spectroscopy, soft-ionization mass spectrometry, and more.

Food Microbiology. Volume I: Concepts in Physiology and Metabolism; Volume II: New and Emerging Technologies. Edited by Thomas J. Montville. Published by CRC Press, Inc., 2000 Corporate Blvd, NW, Boca Raton, FL 33431, due 1986. Vol. I, 224 pp.; Vol. II, 256 pp. Price: Vol. I: $98.00 United States; $113.00 outside United States; Vol. II: $110.00 United States; $126.00 outside United States. ISBN Vol I. 0-8493-6478-7; Vol II. 0-8493-6479-5.

This 2-volume reference delineates the molecular and biochemical principles which are the basis of microbial growth in foods and also presents the underlying concepts for current and future food microbiology practice.

Proceedings of the Seventeenth International Symposium on Free Radicals. Cosponsored by National Bureau of Standards, Granby, CO, August 1985. Available from Superintendent of Documents, U.S. Government Printing Office, Washington, DC 20402. Price: $30.00 prepaid. Stock No. 003-003-02742-1.

Topics at this symposium included laser, infrared, ultraviolet, optical, rotational, vibrational, fluorescence, laser magnetic resonance, laser-induced fluorescence, and other forms of spectroscopy; chemical kinetics and reaction rates for many processes; photodissociation cross sections; radio detection and observations of interstellar species; and other related subjects.

INSTRUCTIONS TO AUTHORS

Scope of Articles and Review Process

The *Journal of the AOAC* publishes articles that present, within the fields of interest of the Association: unpublished original research; new methods; further studies of previously published methods; background work leading to development of methods; compilations of authentic data of composition; monitoring data on pesticide, metal, and industrial chemical contaminants in food, tissues, and the environment; technical communications, cautionary notes, and comments on techniques, apparatus, and reagents; invited reviews and features. Emphasis is on research and development of precise, accurate, sensitive methods for analysis of foods, food additives, supplements and contaminants, cosmetics, drugs, toxins, hazardous substances, pesticides, feeds, fertilizers, and the environment. The usual review process is as follows: (*1*) AOAC editorial office transmits each submitted paper to appropriate subject matter editor, who solicits peer reviews; (*2*) editor returns paper to author for revision in response to reviewers' comments; editor accepts or rejects revision and returns paper to AOAC editorial office; (*3*) AOAC editorial staff edits accepted papers, returns them to authors for approval, and transmits approved manuscripts to typesetter; (*4*) typesetter sends page proofs to author for final approval.

General Information

Follow these instructions closely; doing so will save time and revision. For all questions of format and style not addressed in these instructions, consult recent issue of *Journal* or current edition of *Council of Biology Editors Style Manual.*

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   Introduction. Include information on why work was done, previous work done, use of compound or process being studied.

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   JOURNAL ARTICLE REFERENCE

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

   BOOK CHAPTER REFERENCE

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

   BOOK REFERENCE

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

   OFFICIAL METHODS REFERENCE

   (4) *Official Methods of Analysis* (1984) 14th Ed., AOAC, Arlington, VA, secs 29.070–29.072

4. **Figure captions** (separate sheet(s), **double spaced**): Designate all illustrations, including schemes, as figures and include caption for every one. Identify curves (See **Figures**) and include all supplementary information in caption rather than on face of figure. Spell out word Figure.
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Abbreviation for liter is L; abbreviation for micron is μm. Do not italicize common Latin expressions such as et al. and in vitro; for nomenclature of spectrophotometry, gas chromatography, and liquid chromatography, follow practice of American Society for Testing and Materials.

1/87

AUTHORS IN THIS ISSUE

REVIEW OF URIC ACID METHODOLOGY

Analytical Methods for Measuring Uric Acid in Biological Samples and Food Products

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During the last 7 decades, uric acid methodology has kept pace with the introduction of state-of-the-art technology (e.g., spectroscopy, electrochemistry, chromatography) or the discovery of unique chemical processes (e.g., redox, enzymatic). We envision this practice will continue in the future. There never will be a single analytical method applicable for biofluids or foodstuffs. Therefore, it is imperative that the analyst not only understand the advantages and disadvantages of a procedure, but also thoroughly understand its underlying chemical and technological principles. Since many procedures available for analysis of biofluids and foodstuffs rely on identical chemical or technological principles, this report shall review both sample types and the available spectroscopic, electroanalytical, and chromatographic methods.

Uric acid (7,9-dihydro-1*H*-purine-2,6,8(3*H*)-trione) is excreted as the principal end product of purine metabolism. All mammals except the primates are uricolytic organisms, which possess the enzyme uricase that converts uric acid to the more soluble compound, allantoin. Primates, reptiles, fowl, and insects are nonuricolytic organisms, which excrete uric acid as the major product of purine catabolism (Figure 1). Clinical disorders associated with uric acid stem from increased catabolism or dysfunction of one of the shunt pathways which leads to increased urate production. Diagnosis is confirmed by monitoring plasma or urinary urate levels. Normal serum levels range from 25 to 75 μg/mL, while urinary excretion is typically 250 to 750 mg/day. For years, solubility of uric acid was considered to be its most important physical chemical property. Excessive production of uric acid may lead to precipitation in the lower extremities and joints. For many years, its clinical significance was solely attributed to the metabolic disorder known as gout. More recently, uric acid has been linked to the Lesch-Nyhan syndrome which is an x-linked chromosome disorder that results in the absence of the regulatory enzyme hypoxanthine-guanosine phosphoribosyl transferase (HGPRT).

The presence of insect fragments or excreta in food products is a major concern of government agencies which regulate the food processing industry. Uric acid is stored in the body fat of insects, circulated in the blood, and excreted as a major product of protein and purine metabolism (1). Uric acid levels are used to estimate urinary and fecal nitrogen in poultry excreta (2). Anderson and Patton (1) have postulated that uric acid levels in insect body fat reflect protein metabolism because the levels decrease during food deprivation and increase with a high protein diet. Large amounts of uric acid exist even when uricase is present. For example, blood levels are about 230 mg/L in the adult cricket, *Acheta domestica*, and about 150 mg/L for crickets in the larval stage (3). Uric acid is normally absent in grain and vegetable material and therefore serves as an ideal chemical indicator for filth or insect infestation. Duggan utilized the uric acid content of egg products to estimate chicken manure contamination (4). Likewise, this chemical marker can indicate the degree of infestation in grains and fruit (5, 6). Measurement of uric acid levels has not only aided in the diagnosis of clinical disorders and the presence of filth and infestation in food products but has also aided in entomological studies. For example, uric acid levels have helped determine food utilization by several insect species (7, 8) and uric acid excretion rates of the rust-red flour beetle (9).

Since 1894, numerous publications have appeared describing methods for uric acid analysis in clinical, food, and entomological studies. Introduction of these methods closely parallels the discovery and application of new chemical reaction technology. Today, the analyst has an entire array of different methodologies to apply to a problem. Normally, analytical procedures are first developed for clinical samples, then adapted for entomological and filth-infestation of foods. Several reviews for biological samples (10–13) and a review for biological samples, food, milk, and avian excreta have also appeared (14).

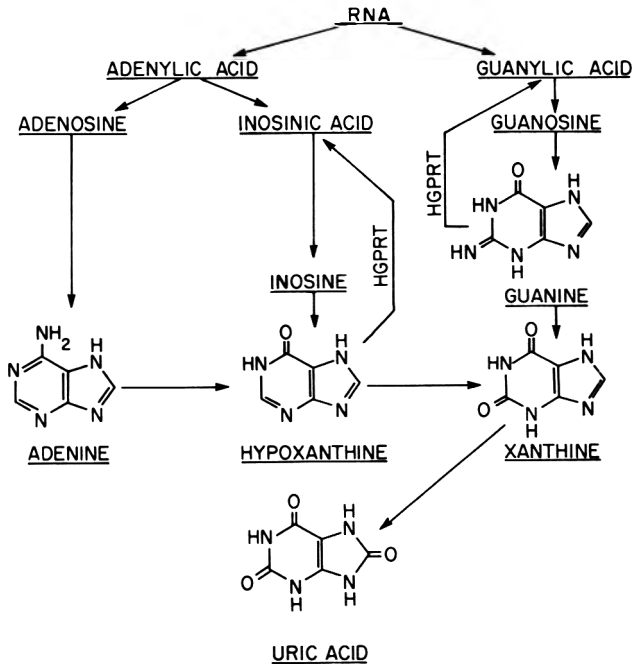

Figure 1. Biosynthesis of uric acid.

Accepted August 7, 1986.

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This review surveys the analysis of uric acid in clinical samples, food products, and entomological samples. Stability and sample isolation techniques have not been included because they are discussed in the cited papers. Rather, we have focused on the chemistry or techniques associated with uric acid methodology. Only selected papers that are of historical importance, important to the chemical understanding of the method, or important in advancing the selective, sensitive analysis for uric acid have been cited. The review is divided into 3 major sections: spectroscopic, electrochemical, and chromatographic methods.

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Spectroscopic Methods

The chemistry associated with spectroscopic analysis for uric acid can be divided into 2 main classifications: redox-colorimetric and enzymatic. Both categories are based on oxidative degradation of uric acid and are popular because they use inexpensive reagents and the technique is simple. Spectroscopic methods account for better than 80% of the practical assays.

Redox-Colorimetric Methods

Redox indicator methods rely on the ease of uric acid oxidation in acid or alkaline media. Mild oxidizing agents convert uric acid to alloxan and urea in acid solution, whereas in alkaline media, the products are allantoin and carbon dioxide (Figure 2). These methods can be further subdivided into phosphotungstate, metal ions, and miscellaneous.

Phosphotungstate.—This method remains the most popular colorimetric method because it is straightforward and uses inexpensive chemicals. In 1894, Offer (15) reported the production of a blue product when uric acid reacted with phosphotungstic acid. Folin and Macallum (16) were the first to apply this reaction for monitoring urinary urate (17); Folin and Denis (18) monitored serum urate. The phosphotungstic method is based on the reduction of phospho-18-tungstic acid to tungsten blue, allantoin, and carbon dioxide. The absorbance between 660 and 720 nm is proportional to uric acid concentration. Uric acid determinations in biological fluids are performed in plasma and serum because glutathione and ergothioneine from red blood cells interfere. Several endogenous interferences include: ascorbic acid, cysteine, glutathione, gentisic acid, tryptophan, and other reducing agents that cause positive biases. Negative biases result from poor recovery or color instability. Folin and Marenzi in 1929 (19) and Folin in 3 subsequent papers (20–22) proposed several modifications to improve sensitivity and selectivity. In his last paper (22), he stated, "The colorimetric method for the determination of uric acid represents probably the most complex reaction in the whole field of colorimetry."

For 7 decades, research has largely been directed at improving specificity, sensitivity, color stability, or decreasing turbidity. Caraway (23) obtained optimal color stability when pH was increased with Na_2CO_3. Optimal color development occurred at 30 min, but if the pH was further increased, color instability occurred. Henry et al. (24) evaluated Caraway's method and determined that uric acid recovery was minimized if the deproteinized filtrate pH was less than 3. The original Caraway method has been rigorously evaluated by independent investigators and chosen as a selected method for clinical laboratories (12). Potential interferences have been documented (25).

An automated method which involves dialysis has been described (26) that gives higher recoveries than the manual Caraway method. Brown and Freier (27) used improved flow cells, dialysis membranes, and an alkali incubation delay coil to optimize sensitivity and specificity over earlier methods (26, 28). Two semi-micro adaptations of the Henry method have appeared (29, 30). Both procedures required 0.2 mL plasma and minimal sample manipulation and gave results

comparable to those of an earlier procedure (24). The methods are linear to 0.2 mg/mL with precisions of ±5%.

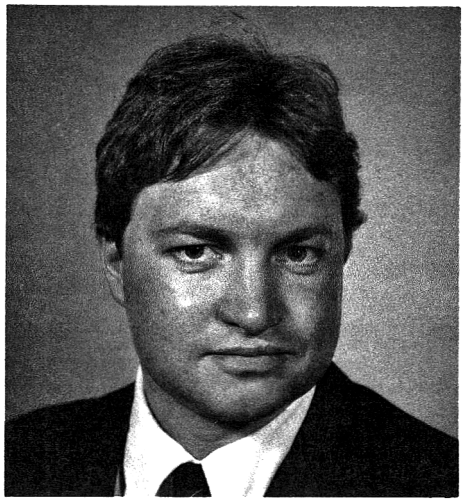
Archibald (28) proposed that the phosphotungstate reagent could be used for deproteinization and oxidation. This concept is routinely used in contemporary methods to minimize sample preparation steps. An automated version has been described (31) that retains the same reliability and specificity. In this method, the sample stream was diluted with NaOH (to destroy nonurate chromogens), neutralized with HCl, and dialyzed at a sampling rate of 40/h. The dialysate was mixed with a sodium silicate–glycerol solution to prevent turbidity.

Two popular methods (32, 33) were routinely used before development of the Caraway or Archibald methods. Their popularity resulted from an increase in sensitivity when NaCN was used for alkalinization. However, these methods are considered too dangerous for the modern laboratory. Sobrinho-Simoes and Pereira (34) substituted hydroxylamine as the alkaline agent and obtained comparable sensitivities. Beer's Law was obeyed up to 0.08 μg/mL. The reaction product was stable, and endogenous compounds (glucose, ascorbic acid, cysteine, and salicylates) did not interfere. An automated version using a dialysis module and an oxidative reaction coil was introduced by Musser and Ortigoza (35). Their procedure was simple, specific, and linear up to 4 mg/mL with a sensitivity comparable to that of sodium cyanide methods. The following reagents have been used to increase sensitivity and reduce sample turbidity: EDTA–hydrazine (36), sodium glycinate (37), and a triethanolamine–carbonate–urea mixture (38).

Several procedures have been introduced for monitoring uric acid in contaminated food products (39–41). Laessig et al. introduced an automated method for determining low uric acid levels in dry milk, flours, and cereal grains (39). This method was based on the sensitive cyanide method of Brown (33) and Benedict and Hitchcock (32) which used a dialysis membrane to selectively isolate uric acid. Macro (1 g) or micro (0.1 g) samples could be assayed with a linearity range of 0.04 to 16 mg uric acid/g product. The mean recovery was 104%, and 30–40 samples/h could be processed. A semiautomatic differential colorimetric procedure for determining uric acid in insect-infested food was introduced by Roy and Kvenberg (40). True sample blanks were obtained after incubation with immobilized uricase. Samples were analyzed using a hydroxylamine–borate–phosphotungstate procedure. The method offered 2 major advantages over Laessig's procedure: highly colored samples could be processed, and the reaction was more specific because of uricase incubation. An automated procedure that allowed processing a more representative (50 g) sample with improved sensitivity (0.1 mg/100 g), excellent recoveries (97.1%), and correction for the presence of a flour maturing agent (potassium bromate) has been described by Galacci (41).

Metal ions.—Another major redox class involves the stoichiometric reduction of metal ions to produce a stable colored product. The common metal ion redox methods involve the reduction of iron(III) to iron(II). An intensely colored iron(II) complex is formed after addition of a chelating agent, and the absorbance of the complex is directly proportional to the uric acid concentration. The most common iron(II) chelating agents are: 1,10-phenanthroline, 2,4,6-tripyridyl-*s*-triazine (TPTZ), and 5-pyridyl benzodiazepin-2-one.

Morin and Prox (42) published a manual micromethod for serum urate (0.1 mL of sample; λmax. = 505 nm). The method was linear to 0.18 mg/mL with a precision of 2.08% and a recovery of 99.3%. An automated version has appeared that minimizes ascorbate interference and increases the linearity range to 0.20 mg/mL (43). Recent reports indicate that thymol, sodium azide, and the catecholamines interfere (44–46).



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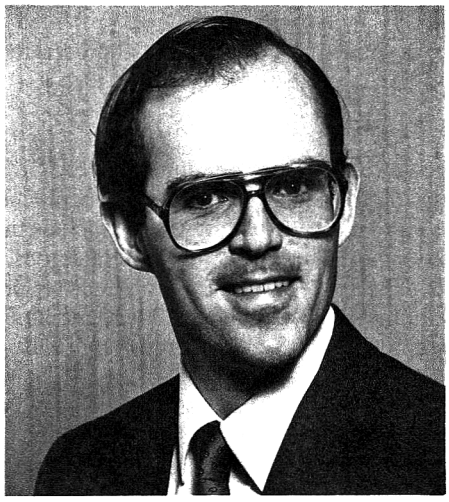
Eswara Dutt and Mottola (47) described a kinetic procedure based on the pseudo-induction period that occurs when iron(II)–phenanthroline complex is oxidized by chromi-



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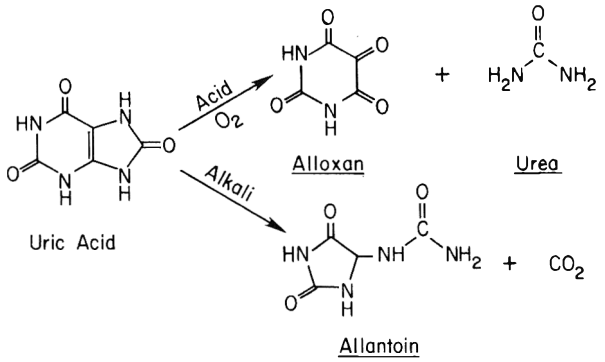

Figure 2. Oxidative degradation of uric acid in acidic and alkaline solutions.

um(VI) in the presence of oxalic acid. The *o*-phenanthroline–ferrous complex has also been used as an indicator for the ceric sulfate titrimetric determination of uric acid in avian excreta (14).

A serum micromethod (20 μL) which offers a 2-fold increase in sensitivity has been described using 2,4,6-tripyridyl-*s*-triazine (λmax. = 593 nm) (48). 5-Pyridyl benzodiazepin-2-one has been employed as a chelometric agent (49). A kinetic blank correction version of the manual TPTZ method has also appeared (50). The kinetics of the reaction between iron(II) and ferrozine, 1,10-phenanthroline, and a,a′-bipyridine have been studied (51). Other variations of chelometric methodology for biofluids involve the use of Cu(II) and 1,10-phenanthroline (52, 53), 2,2′-bicinchoninate (54), or neocupriene (55).

Miscellaneous.—The arsenotungsate method of Benedict and Franke (56) found widespread use during the first half of the 20th century for biofluids or infested foodstuffs (57). Other methods include the analysis for uric acid using potassium ferricyanide (58) or a differential procedure that uses uricase and ferricyanide (59).

Enzymatic Methods

The specific oxidative enzyme uricase (E.C. 1.7.3.3) has been widely applied to the analysis of body fluids and infested foodstuffs. The 3 major enzymatic classes include the uricase, uricase–peroxidase, and uricase–catalase methods.

Uricase.—The single-step uricase methods are popular for biological fluids and food products. These methods monitor the decrease in absorbance (292 nm) which results from the oxidative destruction of uric acid (Figure 3). The reaction is specific, but high background absorbance and enzyme inhibition by several purines may yield poor reproducibility (60).

A practical differential enzymatic spectrophotometric method was introduced by Kalckar (61). This procedure was further characterized and applied to the determination of uric acid in urine, blood, and spinal fluid using purified laboratory (62) and commercially available uricase preparations (63). Feichtmeir and Wrenn (64) reduced absorbance contributions from plasma protein and uricase via trichloroacetic acid deproteinization; classical column chromatography has also been useful for minimizing these biases (65, 66). Remp (67) introduced a selected method for clinical chemists which was rigorously evaluated and found suitable for routine clinical analysis. His method combined the enzymatic specificity of the Praetorius methods (62, 63) with clean deproteinized filtrates using perchloric acid.

Increased sample throughput, improved accuracy, and improved precision has been realized by the introduction of sophisticated instrumentation. Praetorius methods were automated by Barron and Bouley on the Technicon AutoAnalyzer (68). Duplicate sample analysis enabled a mean recovery of about 100%, sample throughput of 20/h, and a linearity range of 0.02 to 0.20 mg/mL. Steele's modification (69) increased the sample analysis rate to 30 samples/h. His procedure incorporated segmented continuous flow, isolation of uric acid from the sample by dialysis, and simultaneous incubation with and without uricase. Another modified AutoAnalyzer version has also appeared (70).

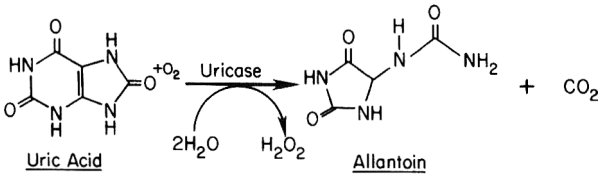

Figure 3. Enzymatic degradation of uric acid by uricase.

The automated method most similar to the manual Praetorius methods was introduced by Pesce et al. (71) and was based on the centrifugal analyzer. The underlying principles are simple. An initial absorbance reading at 293 nm is obtained 3.0 s after starting the rotor. At this time, uricase has not measurably oxidized uric acid, thus the initial absorbance approximates the reading that would be obtained if only the reagents were present. After 5.0 min the reaction is complete, and the difference in absorbance is proportional to uric acid concentration. A major advantage is that blanking of the sample and the uricase reaction is obtained in the same cuvet. Guilbault and Hodapp (72) proposed a direct reaction rate fluorometric method for the quantitation of uric acid. The peroxide formed from the uricase reaction reacts with phthalic anhydride to form peroxy phthalate, which can be oxidized to a highly fluorescent dye (λexcitation = 395 nm, λemission = 470 nm). Their method was linear from 0.50 to 25 μg/mL with an accuracy and precision of about 2%.

Priest and Pitts (73) studied the uricase reaction in borate systems and concluded that gross inaccuracies may be observed if the UV spectrum of a uricase reaction intermediate is not accounted for. They recommended use of a 0.05M borate buffer to eliminate this interference. Tawa et al. (74) proposed a sensitive and accurate UV assay procedure which accounted for the reaction intermediate. Their method had acceptable linearity, recovery, and precision. They reported a Michaelis constant of 8.06×10^{-6}M for the enzymatic oxidation.

A modified Kalckar procedure was introduced by Farn et al. (75) and Farn and Smith (76) for food products (10–320 mg uric acid/100 g). The method involved extraction with 5% sodium acetate, uricase incubation, protein precipitation with trichloroacetic acid, and monitoring of the differential absorbance (λmax. = 284 nm). An improved procedure was reported (77) which minimizes formation of turbid solutions, eliminates protein precipitation, destroys the "uricase activity" in samples, and improves reproducibility. The lower limit of quantitation was lowered to 3.2 mg/100 g of sample, and the method was applicable to wheat flour, rye flour, rice flour, corn meal, oatmeal, barley, and infant soy cereals. At the 1966 AOAC meeting, the Associate Referee recommended that the method sensitivity be improved and uric acid levels in infested flour samples be correlated with existing microscopic methods. Sen (78) introduced a modified method which used a methanolic–LiOH extract to lower the detection limit to 50–60 μg uric acid/100 g. Preliminary results indicated excellent correlation between uric acid levels, insect fragment counts, and uric acid excretion rate by larvae,

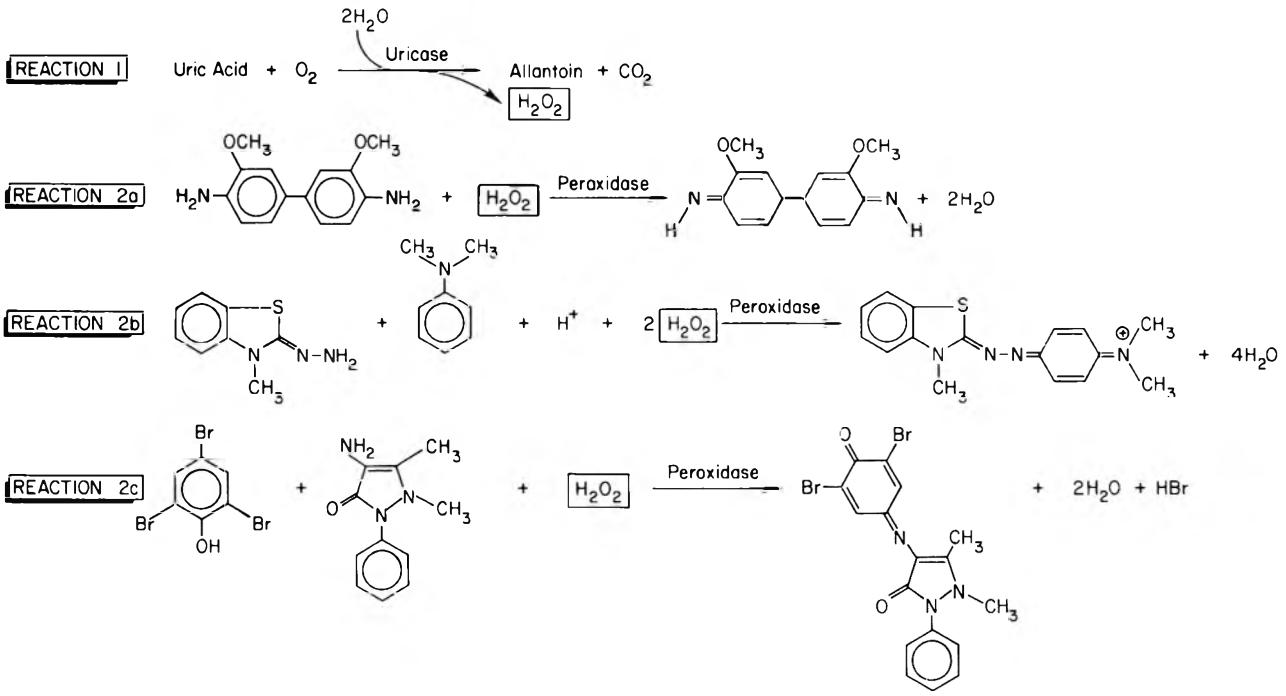

Figure 4. Overall reaction for the uricase peroxidase methods. Reaction 1: Enzymatic degradation of uric acid; reaction 2a: peroxidase-*o*-dianisidine oxidative couple; reaction 2b: peroxidase-MBTH coupling reaction; reaction 2c: peroxidase-aminoantipyrine coupling reaction.

pupae, and adults of the insect *Tribolium castaneum* Herbst. Pudelkiewicz et al. (79) have also reported an Li_2CO_3 procedure (without methanol) to ascertain the uric acid content of avian excreta. Sen's method (78) has been applied with minimal modification for estimating the degree of chalcid infestation in alfalfa seed (80).

Uricase–peroxidase.—Specific and sensitive methods have been developed utilizing the hydrogen peroxide produced from uricase. Hydrogen peroxide oxidizes chromogenic dyes or facilitates oxidative coupling reactions. Both reactions are catalyzed by peroxidase (E.C. 1.11.1.7). The absorbance of the chromophoric agent is directly related to the original uric acid content. The chromogenic reagents include: *o*-dianisidine, 3-methyl-2-benzothiazolinone, aminoantipyrine, and miscellaneous reagents.

Lorentz and Berndt (81) used *o*-dianisidine as the chromophore. The hydrogen peroxide formed from the uricase reaction oxidized *o*-dianisidine (pH 8.5) to a brownish quinonediimine (Figure 4). The absorbance of the purple color was measured at 530 nm within 30 min after the addition of 9N sulfuric acid. Domagk and Schlicke (82) used a modified version which destroyed endogenous serum catalase activity via ethanol deproteinization, and minimized uricase inhibition by using a strongly buffered reaction medium. An automatable procedure has been introduced that monitors the absorbance change at 436 nm and is linear up to 0.20 mg/mL (83).

The oxidative coupling of H_2O_2 with 3-methyl-2-benzothiazolinone-*N,N*-dimethylaniline (MBTH) is the basis for some methods (Figure 4). An automated method has been reported (84) which requires dialysis for improved specificity and has a linearity range of 0.005–0.10 mg/mL. Another procedure incorporated an immobilized uricase column into the AutoAnalyzer flow system (85). In this method, serum uric acid was dialyzed and oxidatively decarboxylated by the immobilized enzyme, and H_2O_2 was reacted with horseradish peroxidase and MBTH–*N,N*-dimethylaniline. The column was stable for 2000 continuous determinations with a sample throughput of 50 samples/h. This report provided directions for enzyme immobilization and incorporation into the flow system.

Recent biofluid methods are based on solid phase reagents and the Ames Seralyzer (86–88). This new technology incorporates standard chemical reagents onto cellulose strips. Serum samples (about 30 μL) are spotted onto the strip, incubated, then quantitated using reflectance spectroscopy. A method that uses the uricase–peroxidase–MBTH–primaquine phosphate system has been introduced (89). The method, requiring only 2 min for reaction, was linear from 0.002 to 0.16 mg/mL with a precision of 5.2% for a 0.05 mg uric acid /mL sample. Potential applications and pitfalls of solid phase methodology have been discussed (90).

Other methods are based on the oxidative coupling of a phenolic reagent to 4-aminoantipyrine. Kabasakalian and coworkers (91) described a manual method which monitored the reaction between 2,4,6-tribromophenol and 4-aminoantipyrine at pH 7.0 (Figure 4). Their procedure required serum deproteinization, and the pH was modified during the reaction to individually optimize the uricase and peroxidase enzyme systems. Absorbance (492 nm) was proportional to the original serum urate concentration up to 200 μg uric acid/mL with a precision of 2.6%. Advantages included: color stability, minimal interference by reducing substances (except glutathione), and the use of noncarcinogenic reagents. An automatable differential kinetic method which substitutes *p*-hydroxybenzoate as the phenolic reagent has been introduced (92). Unlike previous methods, the dual enzyme method is performed at a single pH (7.0). The difference in absorbance obtained at 0.33 and 5 min is proportional to serum urate. The method is linear up to 0.12 mg/mL with a recovery of about 100% and a precision of less than 2.2%. Advantages of this method included elimination of deproteinization, long reaction times, sample blanks, and use of carcinogenic reagents.

The automated oxidative coupling of 2,4-dichlorophenol with aminoantipyrine has been described by Klose et al. (93). 2,4-Dichlorophenol was chosen because the molar absorptivity of the reaction product (11.5×10^6 cm²/mol) was ap-

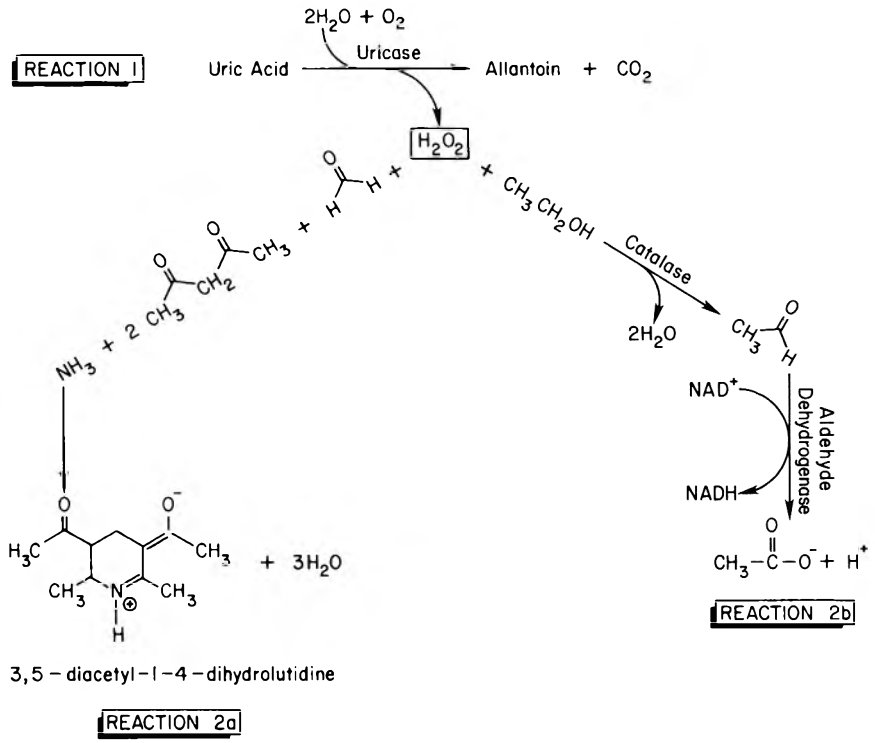

Figure 5. Overall reaction for the uricase-catalase methods. Reaction 1: Enzymatic degradation of uric acid; reaction 2a: Kageyama (103) uricase-catalase enzymatic couple; reaction 2b: Haeckel (106) uricase-catalase-aldehyde dehydrogenase couple.

proximately 2-fold greater than that achieved with phenol. Optimal aminoantipyrine and 2,4-dichlorophenol concentrations of 3.6 and 3.0mM were recommended to obtain a sensitive method which could process 60 samples/h. Interference by hemoglobin, bilirubin, or lipemia were minimized by dialysis. This method has been adapted to the SMA II analyzer by Sewell (94). Satisfactory sensitivity, recovery, linearity, and precision were obtained.

A flow system incorporating immobilized uricase and the peroxidase 2,4-dichorophenol antipyrine system has been introduced by Sundaram and coworkers (95). This report described the preparation, characterization, storage, and incorporation of the immobilized enzyme into the flow system. The preparation was stable for at least 3 months or 4000 determinations. Apparent K_m values for immobilized uricase ranged from 0.06 to 1.0mM, a range higher than the homogenous value (0.02mM). This technology offers reduced sample analysis cost by enzyme reuse.

A major disadvantage of the "phenol–aminoantipyrine" reaction is that the optimal pH for uricase is not identical for horseradish peroxidase. Fossati and colleagues (96) proposed that bacterial uricase can be substituted for animal uricase and 3,5-dichloro-2-hydroxybenzene sulfonic acid be used as the phenolic reagent. These investigators concluded that the optimal pH for bacterial uricase was more compatible with horseradish peroxidase. Sulfonated phenol provided a 4-fold absorbance increase over phenol. Other investigations based on a sulfonated dichlorophenol involve removal of interferences by incubation with hexacyanoferrate (97), evaluation of optimal reaction conditions (98), and evaluation of a commercial kit (99).

Miscellaneous reagents which produce fluorescent products include homovanillic acid (100) and *p*-hydroxyphenylacetic acid (101). A report surveying 25 potential indicator substrates for the peroxidase enzyme recommended *p*-hydroxyphenylacetic acid, tyramine, and tyrosine as excellent substrates (102).

Uricase–catalase.—The first application coupling the uricase enzyme with catalase (E.C. 1.11.1.6) was introduced by Kageyama (103) (Figure 5). The absorbance of 3,5-diacetyldihydrolutidine was monitored at 410 nm. The method does not require deproteinization and is linear to 0.2 mg/mL with recoveries of 100.6% and a precision of 1.2%. Kageyama also investigated the optimal absorption maxima, product stability, and influences of bilirubin, ascorbic acid, and glucose. The method was easier but gave results similar to those of a uricase UV procedure. Mertz (104) has confirmed these results and has recommended this simple, accurate procedure for routine use in the clinical laboratory. An automated version has been reported (105).

Haeckel (106) introduced the first tricoupled enzymatic determination for urinary and serum urate. This methodology utilized the uricase, catalase, and aldehyde dehydrogenase (E.C. 1.2.1.5) enzymes. The reaction sequence is given in Figure 5. The absorbance of NADH produced in the reaction was monitored near 334 nm, and method specificity was defined for 60 drugs. The method is more sensitive than the standard uricase procedure and is more specific and faster than the Kageyama procedure. These observations have been confirmed by Trivedi (107). Although kinetic methods for the measurement of substrates provide a high degree of accuracy, the shortened reaction time normally decreases sensitivity. The sensitivity increase of the Haeckel method allows it to be easily adapted to automated kinetic determinations (108, 109). This methodology has been adapted for routine use on the Centrifical analyzer by White and coworkers (108). Their method was based on the difference in absorbance at 6 and 210 s. It was linear to 0.16 mg/mL, which is comparable to other available methods. Although their procedure was based on the measurement of NADH, they recommended that endogenous lactate interference could be minimized if NADP+ was substituted as the cofactor. Bartl and coworkers (109) showed that alcohol dehydrogenase isoenzymes released in patients suffering from

acute liver or renal disease cause positive biases. They proposed that pyrazole and oxalate (50–70mM) should be used as inhibitors of these enzymes.

Electroanalytical Methods

Electroanalytical methods for uric acid can be classified into 3 major categories: direct, enzymatic, and miscellaneous. These major classifications and the electrooxidative mechanism of uric acid will be discussed.

Electrooxidative Mechanism

Electrooxidation of uric acid involves a 2e-2H$^+$ electrooxidation to a diimine with subsequent hydration to a 4,5-diol. The electrode mechanism is illustrated in Figure 6. The electrooxidation of uric acid was investigated by Dryhurst (110). The major products of the electrooxidation were dependent on solution pH (111). In acidic solutions (pH 1–3), alloxan and urea were the major products whereas at higher pH the major product was allantoin. More recent reports (112–116) confirm the mechanism set forth by Dryhurst in 1972 and indicate that the electrode mechanism closely parallels the enzymatic degradation of uric acid at physiological pH values (116).

The monoanionic form of uric acid exists at neutral pH (pKa = 5.75) (115). The monoanion is oxidized (2e-2H$^+$) to a labile diimine anion (II). The peak oxidation potential is pH-dependent according to the following equation: Ep (pH 0–11.5) = (0.685 − 0.055 pH) V vs SCE (25°C). Components I and II form a quasi-reversible couple, and the cathodic peak can be observed with a rough pyrolytic graphite electrode at sweep rates greater than 200 mV/s; smooth or highly polished electrode surfaces require much higher sweep rates (>20 V/s) to observe the cathodic peak (114). The very unstable diimine anion (II) is hydrated in 2 steps. The first is a rapid hydration characterized by a pseudo first order rate constant of 32.5/s at pH 8 (phosphate buffer, 0.5M ionic strength); this corresponds to a half-life for II of 21 ms (112). At both lower and higher pH values, the rate of hydration increases. Component II can undergo a 2e-2H$^+$ reduction of the nonhydrated double bond to yield a dihydro derivative (115). The peak potential vs pH relationship for this reduction is: Ep (pH 2.8–10.0) = (−0.50 − 0.066 pH) V vs SCE (25°C) (117). The second hydration reaction (III to IV) is slower with a pseudo first order rate constant of 3.5×10^{-3}/s between pH 7 and 9 (phosphate buffer 0.5M ionic strength) (113); this corresponds to a half-life for III of more than 3 min. Finally, uric acid 4,5-diol (IV) decomposes by way of an isocyanate to allantoin (V) and carbon dioxide in neutral and weakly alkaline solutions (112). Based on these results, Marsh and Dryhurst (113) and Brajter-Toth et al. (115) have demonstrated that both electrochemical and enzymatic (peroxidase) oxidations of uric acid proceed by an identical mechanism.

More recently, Tyagi and Dryhurst (117) reported on the electrochemical and enzymatic oxidations of uric acid in alkaline phosphate solutions. They found a second oxidation peak when high phosphate buffers and alkaline pH were used for electrolysis. Although the intermediates were different, the final products correlated well with the enzymatic oxidation of uric acid.

Direct Methods

Direct methods are rapid and require minimal sample manipulation or reagents; most are voltammetric (the measure of electrochemical current in response to a variable electrode potential). Mueller et al. (118) used in vivo voltammetry at a carbon paste microelectrode to measure the uric acid production by rat caudate. A peak potential of 340 mV vs Ag/AgCl was selected for uric acid oxidation and 0.4 nmol uric acid/mg rat caudate was found. The authors commented that their methodology may provide insight into the Lesch-Nyhan syndrome.

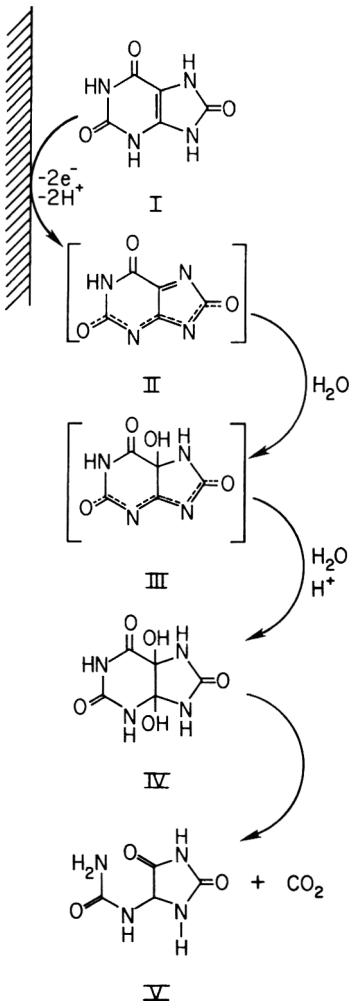

Figure 6. Mechanism for the electrochemical oxidation of uric acid.

Yao et al. (119) described a linear sweep voltammetric method for the simultaneous determination of uric acid, xanthine, and hypoxanthine. Sulfuric acid (1M) served as the supporting electrolyte which minimized adsorption at the glassy carbon electrode surface and provided a 400 mV separation between the anodic peak potentials of each compound. A linear relationship between anodic peak current and concentration for each analyte (0.02–0.4mM) was obtained. Dryhurst and De (120) devised an accurate, rapid voltammetric method for allopurinol and uric acid. Allopurinol (0.1–1mM) was first determined by its reduction wave at the dropping mercury electrode over a pH range of 0–6; the solution was then saturated with allopurinol (7mM), and uric acid (0.05–0.5mM) was determined by its oxidative wave at the pyrolytic graphite electrode. Allopurinol saturation obviated uric acid adsorption to the pyrolytic graphite electrode.

Wang and Freiha (121) improved sensitivity and selectivity of voltammetric measurements by preconcentrating uric acid on a carbon paste electrode. The method involved immersing the electrode in a uric acid sample for 15 s and then transferring the electrode to an electrolytic blank solution for

a differential pulse voltammetric measurement. A significant advantage of this medium-exchange procedure is the ability to directly measure analytes in real samples without sample pretreatment. The precision of the medium-exchange procedure was 14% relative standard deviation (RSD). Flow injection analysis was implemented to improve precision (7.1% RSD). The flow injection method was linear over the range of 2.0–10.0 × 10^{-5}M (r = 0.999).

Nonvoltammetric direct methods have also been developed. Janchen et al. (122) devised an automated continuous flow system with amperometric detection; uric acid in serum was oxidized at a platinum electrode covered with a cellulose membrane. A sample analysis rate of 80 samples/h was reported and the system was stable for >2 weeks. Intrarun and interday precision were less than 1.75 and 5.00%, respectively, and the method was accurate within 5%. A rapid electrochemical method based on a discrete charge injection has been introduced (123). The major drawback of this method is that only total oxidizable substances were measured and positive biases could result.

Enzymatic Methods

Electroanalytical methods based on the uricase reaction (Figure 3) utilize either amperometric, potentiometric, or coulometric detection. In these methods, the concentration of uric acid can be quantitated by measuring either the consumption rate of oxygen or the production rate of hydrogen peroxide or carbon dioxide. Temperature control is critical (124), because the enzymatic reaction has an activation energy of 12.4 Kcal/M.

Amperometric.—Most enzymatic methods involve the amperometric measurement of oxygen consumption. Electrodes include the "polarographic" oxygen sensor and immobilized uricase electrodes. "Polarographic" is a misnomer because the dropping mercury electrode is not used. Furthermore, an amperometric measurement (electrochemical current measured in response to a constant electrode potential) is made, not a voltammetric measurement of which polarography is a subset. In these methods, the Clark oxygen electrode (125, 126), which is a platinum cathode covered with an O_2-permeable membrane, is set at −0.5 to −0.6 V vs the Ag/AgCl reference. Current is proportional to the partial pressure of O_2 consumed in the uricase reaction, which is proportional to the original uric acid concentration.

Bell and Ray (127) were first to use the oxygen sensor of the Beckman "Glucose Analyzer" to measure serum and urinary urate. A 50 μL serum sample was injected into a thermostated, stirred sample cup containing 1 mL reagent. The maximum initial velocity was sensed and displayed within 15 s. Sixty samples/h could be processed and assay precision was ±2% at 10 mg/100 mL. Correlation with the phosphotungstic acid–cyanide method was excellent (r = 0.99). Brown et al. (128) used the oxygen electrode to measure uric acid levels in spice products to determine the degree of insect infestation. Meites et al. (129) compared this method to a crystal violet (gentian violet) method and found excellent correlation for plasma samples (r = 0.977). Both ultra-micromethods were acceptable for pediatric samples. Neither method was significantly affected by bilirubin, hemolysis, or normal ascorbate concentrations; however, both methods gave plasma results that were lower than those for a phosphotungstate method. The authors considered the amperometric oxygen method to be superior over the crystal violet method. Within-run precision varied between 3.4 and 9.4%; recovery was greater than 92% and the lower detection limit was 15 mg/mL.

Immobilized uricase electrodes for amperometric O_2 measurement have been developed to conserve enzyme and to facilitate quantitation. Nanjo and Guilbault (130) immobilized uricase onto a platinum electrode using glutaraldehyde and did not require a membrane. The principle of their method is simple: The electrode was inserted into the biofluid, uric acid diffused into the chemically bound uricase and was oxidized, and the amount of O_2 depletion was monitored. After each measurement, the electrode was washed and dipped into a borate buffer solution for 2 min to replenish dissolved O_2 near the electrode surface and to remove reaction products. Both the initial rate and steady-state current approaches were used. The authors discussed the effects of pH, presence of other compounds (ascorbic acid, amino acids, albumin), and applied potential on electrode response. Uric acid determinations in serum and urine correlated with a spectrophotometric method (r = 1.02 and 1.00, respectively). The electrode could be used for 100 days. A different approach was taken by Kulys et al. (131) who used a bienzyme (uricase and peroxidase) amperometric electrode in the presence of hexacyanoferrate(II). The reactions involved are:

$$\text{uric acid} + O_2 + 2H_2O \xrightarrow{\text{uricase}} \text{allantoin} + H_2O_2 + CO_2$$

$$H_2O_2 + 2[Fe(CN)_6]^{-4} + 2H^+ \xrightarrow{\text{peroxidase}} 2[Fe(CN)_6]^{-3} + 2H_2O$$

Hexacyanoferrate(III) was reduced at the glassy carbon electrode at 0.0 V vs Ag/AgCl. The low operating potential offered high selectivity and a linearity range of 0.005–0.13mM uric acid.

Rather than use bulk uricase solutions or uricase immobilized electrodes, other investigators have developed immobilized uricase reactors for use in continuous flow systems. Iob and Mottola (132) immobilized uricase with glutaraldehyde onto controlled-pore glass (CPG-3000), which was used as a packing in mixing-delay coils for continuous-flow uric acid determinations. The rate of oxygen consumption was monitored by a 3-electrode amperometric system (platinum working and auxiliary electrodes, SCE reference electrode, $E_{applied}$ = −0.60 V vs SCE). The instrumental setup has been detailed by Wolff and Mottola (133). Their application also included catalase to remove interference by H_2O_2.

$$\text{uric acid} + \tfrac{1}{2}O_2 \xrightarrow[H_2O]{\text{uricase + catalase}} \text{allantoin} + CO_2$$

Several important features of this method include: a sample throughput of 100 samples/h, a precision of <4% RSD, and linear calibration ranges from 0 to 10 and 10 to 100 mg uric acid /100 mL biofluid; over 70% of the enzyme activity was maintained after repetitive use of more than 10 months. Good correlation (r = 0.98) was obtained when the method was compared with a commercially available phosphotungstic acid method.

Recently, Iob and Mottola (134) described a procedure for immobilizing uricase to "whisker" (filament) surfaces in glass capillary columns; glutaraldehyde and acetone solution of 3-aminopropyltriethoxysilane were used. Immobilized uricase coils (31-turn) have been employed in a continuous-flow system to measure uric acid concentrations. The linear concentration range was 1 to 100 mg urate/100 mL solution with a determination rate of over 200 samples/h. Yao et al. (135) used an immobilized uricase reactor and a peroxidase electrode for the flow injection analysis of uric acid. The hydrogen

peroxide generated in the reactor formed ferricyanide in the presence of the peroxidase electrode. The working reduction potential was −50 mV vs Ag/AgCl. Features include a linear range of 0.2 to 8.0 mg/dL and a determination rate of 120 samples/h with precision of 2% RSD.

Potentiometric.—Kawashima and Rechnitz (136) developed a potentiometric immobilized uricase electrode. The electrode was constructed by coating a uricase slurry onto a Severinghaus-type, gas-sensing pCO_2 electrode and covering the assembly with moistened Cellophane. The potentiometric measurement (potential measurement of nonpolarized electrodes under conditions of zero current) required 5 to 10 min. The linear range was 10^{-4}M to 2.5×10^{-3}M uric acid with a precision of 5% RSD.

Coulometric.—Troy and Purdy (137) devised a constant-current coulometric method (the integral of current over time) for the determination of uric acid in both serum and urine. The method is based on a uricase differential technique, where all reducing substances are measured with coulometrically generated I_2 both before and after treatment with a uricase–catalase preparation. Tungstic acid was used for protein removal. Method precision was typically 3% RSD for serum samples and 2% RSD for urine samples. This method was compared with phosphotungstate colorimetric and UV-enzymatic differential methods. No significant differences were observed between the results on the same or different days.

Miscellaneous.—Barnard (138) described the potentiometric titration of uric acid with potassium ferricyanide. The procedure involved precipitation of urinary uric acid with silver lactate and sedimentation of the precipitate, which was then dissolved with alkali hydroxide–cyanide solution. Potassium ferricyanide was incrementally added until the potentiometric endpoint was reached. This method correlated well with the Folin-Wu method. Kawashima et al. (139) devised a microbial (yeast cells *P. membranaefaciens* 162300) sensor for uric acid. The sensor was constructed by coupling intact uricase-producing yeast cells to the gas-permeable membrane of a carbon dioxide electrode; the membrane was covered with Cellophane to secure the cells. A linear range of 1.0×10^{-4}M to 2.5×10^{-3} mol/dm^3 uric acid was obtained.

Chromatographic Methods

Many of the spectroscopic and electrochemical methods are susceptible to interferences. Therefore, it is not unusual that separation methods have been introduced to increase specificity. The following chromatographic techniques will be discussed in some detail: paper, thin layer, column, gas, and liquid chromatography.

Paper and Thin Layer Chromatography

Numerous paper and thin layer chromatographic (TLC) methods have been reported for the qualitative and quantitative measurement of uric acid. Early paper chromatographic method studies were devoted to finding suitable visualization reagents that could distinguish purine and pyrimidine bases. Vischer and Chargaff (140) precipitated these bases as HgS salts with subsequent conversion to Hg_2S, and a 5 μg detection limit was reported. However, Reguera and Asimov (141) determined that the separation was nonspecific and that spot intensity decreased with time. Their modified version included rinsing the chromatograms with 2% $AgNO_3$, 0.5% $Na_2Cr_2O_7$, 0.5N HNO_3, and water. As little as 0.5 μg purine was detected as red deposits (presumably a purine–$Ag_2Cr_2O_7$ complex), and cytosine, thymine, and uracil did not react.

A method for isolating uric acid from its methylated analogs in human urine appeared in 1952 (142). Samples were fractionated by column chromatography with final separation by paper chromatography. Components were visualized with arsenophosphotungstic acid. The method is capable of separating uric acid, 1-methyluric acid, 3-methyluric acid, 7-methyluric acid, 1,3- and 1,7-dimethyluric acids. A separation method for urinary and plasma ascorbate and urate capable of detecting 0.5 μg/mL has been described (143). Oxidative formation of an iron–phenanthroline chelometric complex was used for visualization. Quantitation was performed by eluting the red chromatographic zones and measuring the absorbance (510 nm).

Caffeine and theophylline metabolism has been evaluated in humans, rats, and rabbits by paper chromatography (144). Uric acid was separated from the methylated uric acid metabolites of these 2 compounds and visualized using urea–cyanide–carbonate–arsenophosphotungstate solutions. Dikstein and coworkers (145) devised a 2-dimensional paper chromatographic procedure which separated substituted xanthines and uric acids. Microgram quantities were detectable, and mercuric complexes were stained with diphenylcarbazone for visualization. Paper chromatography has also been used to monitor the degree of wheat flour infestation by *Tribolium castaneum* Duv. (146). Quantitation was done colorimetrically after elution of the uric acid chromatographic band. Recovery varied between 90 and 100%.

Enhanced uric acid selectivity has been achieved using thin layer chromatography. Pataki (147) resolved 60 nucleo-derivatives by 2-dimensional chromatography on cellulose plates. Several organ extracts were analyzed without sample pretreatment, and uric acid was adequately resolved. Simultaneous TLC determinations of uric acid, xanthine, and hypoxanthine in plasma were described by Orsulak et al. (148). Plasma (1–3 mL) was deproteinized with $LiClO_4$, and the components were separated on cellulose plates. Analyte bands were extracted with 0.05M borax and quantitated by UV spectroscopy. Sample throughput was 12 samples/2 days, and the method was linear to 100 μg. Method precision was ±4.6% with a recovery of 81 ± 4.6%. A TLC method for quantitating uric acid in infested foodstuffs has appeared (149). Pulverized samples were extracted with water, natural colors were adsorbed onto alumina, and uric acid was separated on cellulose plates. As little as 5 μg uric acid could be visualized by arsenophosphotungstic acid and could be quantitated by densitometric or spectroscopic techniques.

Column Chromatography

An ion-exchange chromatographic method for determining uric acid in serum, bile, and urine was published (150). Uric acid was selectively isolated from biofluids on a Dowex 2-X8 column and quantitated by UV spectroscopy, colorimetry, or a uricase procedure. Linearity was observed between 20 and 150 μg/mL, and recovery ranged from 89 to 107%.

Purines and related compounds have been separated by adsorption chromatography on dextran gels (151). Optimal separations were achieved by the judicious choice of mobile phase pH, ionic strength, and flow rate. Reproducible elution volumes for uric acid (about 170 mL) and recovery (99.5 ± 5%) were reported. Uric acid has been resolved from tyrosine, tryptophan, hypoxanthine, xanthine, allopurinol, and oxypurinol on polyacrylamide resins (152). The chromatographic run required 4 h, the column effluent was monitored (280 nm), and the method was linear between 10 and 70 μg uric acid/mL. The retention volume for uric acid was 25 mL, and the method provided precision and specificity similar to

those of enzymatic methods with the convenience of colorimetric procedures.

Gas Chromatography

Chromatographic separations were advanced by the introduction of gas chromatography. This technique provides rapid separations with greater sensitivity and separation efficiency. Its major disadvantage is that derivatization is required to increase volatility of polar compounds.

The trimethylsilylated derivatives of uric acid, bases, and nucleosides have been studied (153). Muni et al. (154) have investigated the chromatographic and spectroscopic properties of clinically relevant trimethysilylated derivatives of purines and pyrimidines. Purines, uric acid, orotic acid, N^6-methyladenine, hypoxanthine, pseudouridine, orotidine, 6-succinopurines, and N^6-methyladenosine were separated and quantitated by dual flame ionization detection on a 3% SE-30 Gas-Chrom Q column. Ultraviolet, infrared, and nuclear magnetic resonance data were reported for uric acid and trimethylsilyl uric acid, and the method was linear between 1 and 5 μg.

GC and LC methods were compared for the simultaneous determination of uric acid, hypoxanthine, xanthine, allopurinol, and oxypurinol (155). Before GC analysis, uric acid was isolated by adsorption chromatography (151, 156) then silylated using bis-trimethylsilyltrifluoroacetamide (BSTFA). The method was linear from 0.125 to 5.0 μg, and excellent correlations between the 2 techniques were obtained for all analytes except allopurinol.

Gas chromatography/mass spectrometry has also been applied to uric acid analysis (157, 158). The chromatographic and mass spectral properties of 6 tetramethyl uric acid isomers have been evaluated (157). On-column methylation with trimethylphenylammonium hydroxide was compared to precolumn methylation with diazomethane. Optimal specificity was obtained by monitoring the m/z 82/83 fragments. This method could be used to monitor ^{13}C or ^{15}N glycine incorporation in patients with purine metabolism disorders. Serum urate determinations were facilitated using tetraethyl derivatives and stable isotope dilution mass spectrometry (158). Ethylation was chosen because endogenous methyl urates may be present. Tetraethyl derivatives were stable and provided unique fragmentation patterns.

Stable isotope techniques have been used to determine the uric acid pool size in spider monkeys (159). Uric acid (1,3-^{15}N) was administered, was isolated from urine by ion exchange, and was derivatized with BSTFA. The percent of ^{15}N urate was determined by measuring the m/z 458/456 fragment ratios. Calibration curves were linear to 25% (^{15}N) uric acid with precisions of ±3.5% and an accuracy of ±7% at >10% incorporation. Ohman (160) used this methodology to evaluate 3 uricase methods. Similar results were obtained when stable isotope techniques were compared to a uricase–UV and a uricase–catalase (103) method. Stable isotope techniques were less accurate than a uricase oxygen consumption method.

Liquid Chromatography

This technique has proven to be an invaluable separation tool for quantitating uric acid in a variety of sample types. Isocratic separations have been reported using ion-exchange, reverse phase, ion-exclusion, adsorption, and ion-pairing techniques. Gradient elution and column switching methods have also appeared.

Ultraviolet detection (LCUV).—Ultraviolet detection of uric acid (280–292 nm) has frequently been used. The early LC reports were confined to ion-exchange stationary phases. Cox et al. (161) quantitated uric acid in animal feeds after extraction with $LiCO_3$, and the method was specific in the presence of xanthine, hypoxanthine, and purines. Linearity was observed between 10 and 140 μg/mL, and the recovery was 98.1 ± 2.2%. A similar micromethod (20 μL) was reported for biological fluids with a linearity range of 0.1–140 μg/mL (162). A simultaneous assay for uric acid and ascorbic acid has appeared that had a minimum detectable limit of 5 μg/mL (163). Topp et al. (164) developed a simultaneous method for monitoring uric acid and pseudouridine in urine and serum. Urine sample preparation consisted of simple dilution with buffer, whereas serum samples were ultrafiltered using microcollodian bags (nominal exclusion = 12 400M). Sample preparation required 4 h, and sample throughput was 12/day. Linearity extended from 0.5 to 15 nmol per injection.

A variety of isocratic reverse phase methods are available. Kiser and coworkers (165) quantitated uric acid in 100 μL serum using a C-18 column. They used adenine as an internal standard to increase precision for this simple deproteinization method. Peak height ratios were proportional to concentration up to 500 μg/mL. Recovery was 102% and day-to-day precision was 2.5%. The following compounds did not interfere: allopurinol, oxypurinol, acetaminophen, salicylate, and theophylline. Putterman and coworkers (155) compared reverse phase and GC analyses of uric acid for its simultaneous determination with hypoxanthine, xanthine, allopurinol, and oxypurinol. The following 3 sample cleanup procedures were also evaluated: (*a*) 5-sulfosalicylic acid precipitation, (*b*) lanthanum nitrate precipitation, and (*c*) a modified Sweetman and Nyhan adsorption isolation (151, 156). These authors concluded that all 3 gave similar results, but the precipitation methods were more rapid.

Wung and Howell (166) reported a method capable of simultaneously measuring 5-fluoruracil, uridine, hypoxanthine, allopurinol, oxypurinol, and uric acid in plasma. Samples were deproteinized with $HClO_4$, and the method was linear over the range 0.4–40 μmol/L. A rapid method (7 min) has appeared for urinary urate and creatinine (167). This liquid-solid isolation method was linear up to 6 mmol/L and had a sensitivity of 2.5 μmol/L. Within-run and between-run precisions were ±3.7 and ±4.5%, respectively, and correlated well with a uricase method.

Reverse phase LC methods have been compared with kinetic and equilibrium uricase methods (168). Serum (100 μL) was deproteinized with acetonitrile, and 20 μL was assayed by LC. Uric acid was quantitatable between 100 and 800 μmol/L, and the LC method was more sensitive than commercial uricase kits. Several purines, ascorbic acid, and the methyl analogs of uric acid did not interfere.

The anaerobic and aerobic degradation of adenine, 6,8-dihydroxypurine, 2-hydroxypurine, hypoxanthine, xanthine, and uric acid has been evaluated by Durre and Andreesen (169) using reverse phase separation. Anaerobic degradation products were optimally resolved at pH 3.5–3.7, while optimal resolution for aerobic degradation products occurred at pH 3.1.

Crawhall et al. (170) adapted methods by Hartwick and coworkers (171, 172) to study xanthinuria and the Lesch-Nyhan syndrome. The modified method consisted of protein precipitation with perchloric acid and detection at 208 nm. This method gave lower uric acid levels than did a uricase procedure (r = 0.79), which was attributed to loss of uric acid during the precipitation step. Bennett et al. (173) intro-

duced another rapid, simple method for urinary and serum urate. Acetonitrile was used for deproteinization and 8-chloro-theophylline was used as the internal standard. Numerous compounds were tested to determine method specificity, and only 2,8-dihydroxyadenine interfered. The method was linear to 1000 μmol/L with a precision of ±4.8–5.5%.

The determination of orotic and uric acid in yogurt cultures has been reported using ion-exclusion chromatography (174). Mizutani and Wada (175) used a porous polymer to isolate and quantitate uric acid in soil samples. This method was applied to determining the isotopic nitrogenous composition of soil in bird rookeries (176).

Metabolic profiles for uric acid, hypoxanthine, and xanthine have been established using paired-ion separations (177). Proteins were precipitated with perchloric acid and resolved from other purines, nucleotides, and nucleosides with 1-heptanesulfonic acid. An efficient ion-pairing system (*t*-butyl counterion) capable of resolving the above analytes and oxypurinol, and allopurinol, has also appeared (178).

Senftleber et al. (179) separated several metabolites and other compounds in uremic patients by using gradient elution chromatography. Hartwick et al. (172) resolved 84 compounds in plasma from normals and neoplasma patients. Amniotic fluid urate levels have been monitored using UV and amperometric detection techniques (180). Nissen (181) introduced a column switching method for measuring allopurinol, oxypurinol, and urate plasma levels. The initial separation was performed on a C-18 precolumn, and the method was linear from 7.5 to 120 μg/mL. Another column switching method has been introduced for monitoring insect infestation in cereals and grains (182) and has been adapted (183) to wheat samples infested with granary weevil (*Sitophilus granarius*), rice weevil (*Sitophilus oryzae*), and lesser grain borers (*Ryzopertha dominica*). Excellent correlations between insect fragment counts and uric acid levels were observed. A detection limit of 1 ppm uric acid corresponding to insect infestation levels of 1 kernel/100 g grain was reported.

Electrochemical detection (LCEC).—The concept of using hydrodynamic amperometric detection for the liquid chromatographic measurement of uric acid was reported by Kissinger and colleagues in 1974 (184). In this feasibility study, diluted urine samples containing uric and ascorbic acids were injected directly onto a strong anion-exchange column, and the effluent was passed through a thin layer amperometric cell. Both compounds were oxidized at a carbon paste electrode, and the oxidative current was directly proportional to analyte concentration. This preliminary work resulted in the development of a serum uric acid method (185). The method consisted of deproteinizing serum (25 or 500 μL) with sodium tungstate followed by LCEC analysis using the original conditions. Alternatively, 2 μL serum aliquots could be assayed after a 1:20 dilution. The method was linear to 250 μg/mL with a detection limit of 100 pg. This method was more specific than a phosphotungstate and uricase procedure (186). Recovery was 100%, and within-run and day-to-day precisions were 0.83 and 1.1% (n = 30), respectively. This method was further modified for ruggedness and specificity (187). Improvements in the method consisted of reducing the working electrode potential to improve specificity and diluting serum samples and injecting the diluent to minimize sample preparation. Over 40 compounds were evaluated and did not interfere. This methodology has been extensively characterized and evaluated by independent investigators and has been designated as a selected method for clinical chemists (188).

Pachla and Kissinger (189) modified their sample preparation steps to adapt the method to monitor insect infestation in cereal and flour products. The detection limit was 2 μg/g, and no interferences from these products were observed. These methods (188, 189) have been applied to determining uric acid renal synthesis in chickens (190) and to determining the net renal reabsorption of urate in the Cebus monkey (191).

Iwamoto et al. (192) have described a reverse phase LCEC method for uric acid in rat serum. A 100 μL aliquot was deproteinized with sulfosalicylic acid and assayed at a thin layer glassy carbon electrode ($E_{applied}$ = +0.8 V vs Ag/AgCl). Other LCEC reverse phase methods have used metaphosphoric acid for deproteinization (193, 194) or have been applied to the quantitation of uric acid in the following sample types: human serum (195, 196), cerebral spinal fluid (196), or human urine (197). The LCEC technique was investigated for the possible determination of hypoxanthine, xanthine, uric acid, allopurinol, and oxypurinol (198). Hypoxanthine and allopurinol were not amenable for amperometric detection because high electrode potentials were required for electrooxidation. Ultraviolet detection was recommended for the simultaneous determination of these compounds.

Various animal tissues have been assayed for uric acid using this technique. A method for measuring uric acid in rabbit brain (199) or the simultaneous measurement of uric acid, ascorbic acid, and the catecholamines (200) has appeared. A report has been published describing the postmortem stability of uric acid in rat tissue (201). Excellent separations of uric and ascorbic acids in serum, urine, and cerebral spinal fluid have been achieved using polymethacrylate (202) and polyvinyl alcohol stationary phases (203). Methods based upon ultrafiltration (204) and alumina adsorption (205) for sample cleanup have been proposed and applied to postmortem brain tissue (206). Other LCEC methods include the use of the wall-jet electrode and dropping mercury electrode (207) or the use of a coulometric detector (208).

Future Directions

During the last 7 decades, uric acid methodology has kept pace with the introduction of state-of-the-art technology (e.g., spectroscopy, electrochemistry, chromatography) and the discovery of unique chemical processes (e.g., redox, enzymatic). We envision this practice will continue. There may never be a single analytical method applicable for biofluids or foodstuffs. Therefore, it is imperative that the analyst not only understand a method's advantages and disadvantages, but also thoroughly understand the underlying chemical and technological principles. A recent publication has exemplified the importance of understanding the stoichiometry of several similar reactions (209). Chemical redox methods shall continue to be important practical laboratory methods. Future substrates in the aminoantipyrine–peroxidase reaction may be found in a 1943 report by Emerson (210) and a 1968 report by Guilbault et al. (102).

Many spectroscopic or electroanalytic methods may not be selective for uric acid; future methods may involve incorporating solid phase isolation columns to enhance specificity. Liquid chromatographic methods may use this technology and may also rely on microbore column technology to improve selectivity and sensitivity. Undoubtedly, robotic sample preparation will be more useful than sample preparation using continuous-flow technology. The major advantage of robotic technology lies in its ability to simulate the manual procedure; it offers truly unattended sample preparation.

Acknowledgment

The authors thank Sandra Robertson for her assistance.

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FOOD ADDITIVES

Determination of Sodium Dioctylsulfosuccinate in Dry Beverage Bases by Liquid Chromatography with Post-Column Ion-Pair Extraction and Absorbance Detection

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Sodium dioctylsulfosuccinate (DSS) is extracted as an ion pair with methylene blue from finished drinks prepared from dry beverage bases. The complex is quantitatively determined colorimetrically in chloroform–acetone solution by a standard procedure. DSS is specifically identified by analyzing an aliquot of the extract by reverse phase liquid chromatography (LC). The compound is detected by using a simple post-column dynamic extraction system in which DSS is extracted from the aqueous mobile phase into chloroform as a methylene blue ion pair. The chloroform phase passes through the absorbance detector for measurement at 546 nm (filter detector). The absolute detection limit was 5–10 ng DSS, while in beverage bases as low as 0.1 μg/g was detected. Extraction of the beverage bases with mobile phase followed by filtration and direct LC analysis with the described system was also successful, although not evaluated on a routine basis.

Sodium dioctylsulfosuccinate (DSS) is a wetting agent permitted as a food additive in a variety of products in the United States and in dry beverage bases in Canada at a level not to exceed 10 ppm in the finished product. Although the compound is not extensively used in Canada, it has recently become of concern as a food additive because of reports of its effects on reproduction (1, 2). The method most commonly used for DSS in such products is a colorimetric analysis described for sulfonated or sulfated surface-active compounds (3) that makes use of the ion-pairing capabilities of these compounds with the dye methylene blue. The approach has been incorporated into the U.S. Food and Drug Administration (FDA) *Food Additives Analytical Manual* for DSS determination (4). However, the method is not specific for DSS but determines total sulfonated or sulfated compounds or, in fact, any substance that forms a chloroform-soluble ion pair with methylene blue. Gas chromatographic (GC) techniques have been used to determine sulfonic acids either after derivatization with diazomethane (5) or by direct analysis with on-column thermal degradation (6). We could not successfully apply the diazomethane reaction to DSS; however, direct injection of DSS produced one predominant peak and a small peak. The large peak was subsequently identified by GC-electron impact mass spectrometry as a thermal degradation product of DSS caused by cleavage of the sulfonate moiety and incorporation of a double bond into the molecule. The reaction was found to be dependent on temperature of the injection port. Application of this approach to the determination of DSS in samples was unsuccessful because coextractives interfered in the thermal decomposition reaction, leading to nonreproducible results.

Three liquid chromatographic (LC) methods have been developed which could be useful for determining DSS in dry beverage base samples. One uses derivatization to form UV-absorbing products of sulfonates, sulfates, or alkyl phosphates (7). We used this method successfully for octane sulfonic acid and hexadecyl sulfonic acid but not for DSS under a variety of reaction conditions. An LC approach involving indirect photometric detection has been reported which includes DSS as one example of the use of the method (8). However, the detection limit was in the low microgram range, and efforts by this laboratory to improve on this were unsuccessful. A post-column, ion-pair extraction technique has been reported for sulfonates and sulfates by both normal phase (9) and reverse phase (10) chromatography with fluorescence detection. The approach appears attractive for DSS analysis because it requires minimum sample preparation and no derivatization, with detectability in the low nanogram range. In applications of the technique to basic compounds in biological fluids using a fluorescent sulfonate as a counter ion, selectivity was very good (11, 12).

The method described in this paper makes use of the post-column, ion-pair extraction principle with several modifications, including the use of a strongly absorbing counter ion, methylene blue, and detection using a common filter absorption detector. The system was constructed completely from commercially available, chemically inert plastic fittings and tubing.

Received March 6, 1986. Accepted May 1, 1986.

METHOD

Reagents

(a) *Solvents.*—Glass distilled.

(b) *Methylene blue.*—Eastman Kodak Methylene Blue Chloride for Biological Staining (dye content, 85%), or equivalent. Prepare 0.001M stock solution in 0.5M H_3PO_4, filter, and store in dark. Prepare working solution by diluting 1 mL stock to 100 mL with 0.5M H_3PO_4.

(c) *Dioctyl sodium sulfosuccinate (DSS).*—Eastman Kodak reagent grade; 96% assay, minimum. Prepare in aqueous solution for recovery studies and in LC mobile phase for analytical standards.

Apparatus

(a) *Liquid chromatograph.*—Waters Model 6000A pump, U6K injector, and Model 440 absorbance detector with 546 nm filter, set to 0.005 absorbance unit full scale.

(b) *Chromatographic column.*—Alltech CN (10 μm, 25 cm × 4.6 mm id). Mobile phase, acetone–0.01M KH_2PO_4 (1 + 4), degassed and filtered; flow rate 1.5 mL/min. Other conditions studied: (*1*) μBondapak CN (5 μm, 30 cm × 4.6 mm id). Mobile phase, acetone–0.01M KH_2PO_4 (2 + 3); flow rate 1.5 mL/min; (*2*) Brownlee C-8 (10 μm, 25 cm × 4.6 mm id). Mobile phase, acetone–0.02M KH_2PO_4 (57 + 43); flow rate 1.5 mL/min; (*3*) CSC (Montreal) C-18 (10 μm, 10 cm × 4.6 mm id). Mobile phase, acetone–0.02M KH_2PO_4 (55 + 45); flow rate 1.5 mL/min.

(c) *Post-column extractor.*—Assemble as shown in Figures 1 and 2 from 3 Hamilton CTFE connectors, fittings, and PTFE tubing (Chromatographic Specialties, Brockville, Canada). Phase separator is 1/16 in. id "Y" connector and eluant mixers are two 1/32 in. id "T" connectors. All tubing is 1 mm id except 10 cm piece of 2 mm id tubing leading from phase separator to waste line. Extraction coil is 50 cm long. Two 1/16 in. stainless steel female union adaptors (Upchurch) connect post-column extractor to LC column and detector. Small

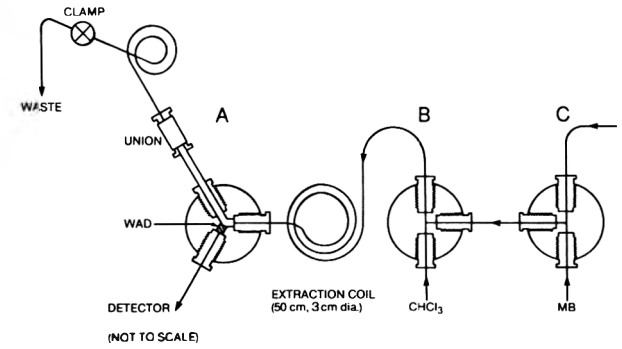

Figure 1. Diagram of post-column extraction apparatus. A, phase separator; B and C, mixing tees. Construction as described in text. MB, methylene blue solution.

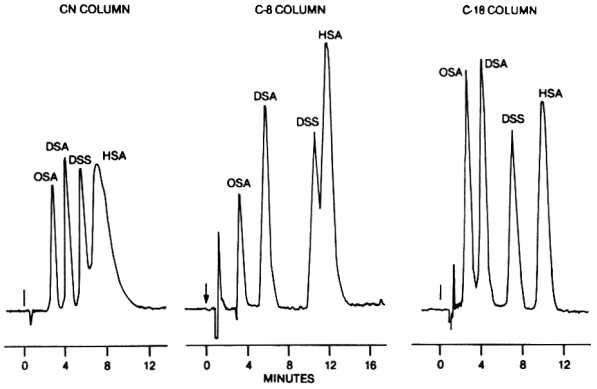

Figure 3. Chromatograms of octane sulfonic acid (OSA), dodecane sulfonic acid (DSA), DSS, and hexadecane sulfonic acid (HSA); 200-500 ng each injected. Columns: CN (Alltech), C-8 (Brownlee), and C-18 (CSC).

hydrophobic solvent-inert filter disc (Millipore, FA, 1.0 μm) is loosely inserted as wad into detector arm of phase separator as shown in Figure 1 to prevent aqueous segments from entering detector but allowing them free passage to waste. Flow rates: $CHCl_3$, 1.0 mL/min using Waters Model M45 pump; methylene blue, 0.1 mL/min, using Technicon AutoAnalyzer proportioning pump; detector, 0.2–0.25 mL/min, adjusted by tightening screw clamp on waste line creating appropriate back pressure.

Always turn on $CHCl_3$ stream first when starting up system, and turn it off last after all aqueous segments have been flushed from system.

Sample Analysis

Colorimetric determination of DSS was performed in samples exactly as described in U.S. FDA *Food Additives Analytical Manual* (4) for dry beverage bases. (Briefly, beverage bases are mixed with water, and an aliquot of preparation is partitioned between chloroform–acetone (1 + 1) and water containing methylene blue. Organic phase containing DSS–methylene blue complex is washed with dilute sulfuric acid and then measured colorimetrically at 657 nm.)

For LC determination, evaporate 5 mL chloroform–acetone solution from colorimetric analysis to dryness under stream of nitrogen at 30°C. Dissolve residue in 0.5 mL LC mobile phase and inject 25 μL of this solution into LC system for analysis. Alternatively, perform direct LC analysis of beverage bases by preparing drinks as directed on packages but using LC mobile phase instead of water. Filter aliquot of mixture (Millex-HV, 0.45 μm, Millipore) and inject 25 μL or other suitable volume into LC system. Quantitate DSS by comparing peak heights or areas to known standards analyzed during same run.

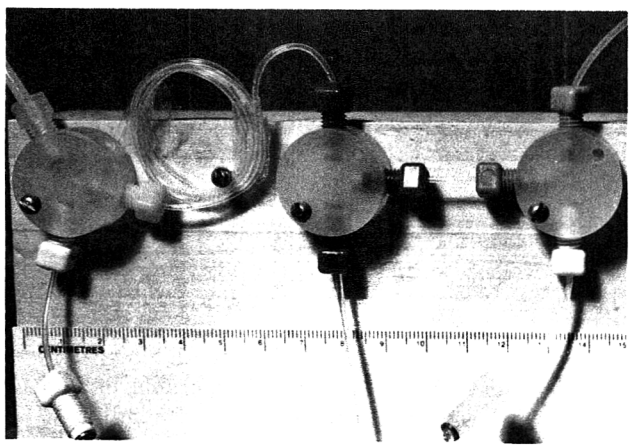

Figure 2. Photograph of post-column apparatus.

Results and Discussion

Post-Column Extraction System

The extraction system was easily constructed from commercially available parts. All parts were of solvent-resistant polymer material, and fittings were tightened by hand. The 3 mixing tees were each mounted vertically in a horizontal line on a block of wood. This is important for the phase separator because the phases separate by gravity. This system was optimized as reported in the literature (11, 12).

Because the Waters 440 detector used in this study created some resistance to flow, it was necessary to apply a back pressure to the system to force the $CHCl_3$ phase through the detector. Back pressure was applied by inserting a screw clamp on the waste line and tightening so that about 0.2–0.25 mL/min of $CHCl_3$ flowed through the detector. A 50 cm length of 1 mm id tubing was also placed between the phase separator exit and the clamp to act as a pulse dampener. This arrangement enabled routine use of the detector at maximum sensitivity (0.005 absorbance unit full scale). Attempting to increase the flow rate beyond 0.3 mL/min through the detector resulted in increased noise attributed to pulsations from the Technicon proportioning pump.

One of the most important factors in the operation of the phase separator is prevention of aqueous phase segments from entering the detector. Recently, membrane phase separators have been designed and evaluated (13, 14) and offer much potential for continuous extraction systems over a range of flow rates and phase–volume ratios. In the present work, however, we found that simply placing a wad of hydrophobic material in the detector arm of the phase separator prevented aqueous segments from inadvertently entering the detector stream. This worked extremely well, and the wad did not have to be replaced at all throughout this study.

The concentration of methylene blue in the post-column system directly affected sensitivity. Using a 0.01mM concentration of methylene blue, the linear range was about 10–250 ng DSS per injection. The range was easily extended by doubling or tripling the dye concentration as required. In practice, the methylene blue concentration was kept as low as possible to minimize background absorbance.

The filter detector used in this work performed very well; however, a wavelength could not be selected to provide monitoring near the absorbance maximum of methylene blue at 657 nm. Although 546 nm was used and was less sensitive

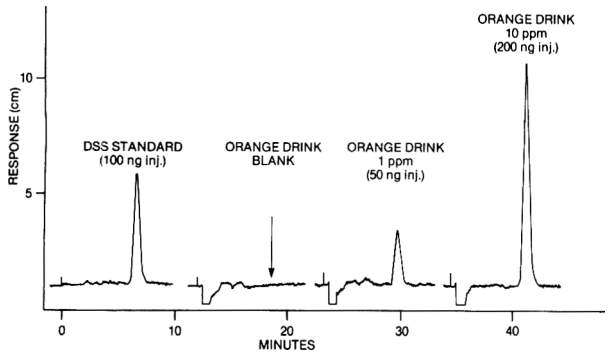

Figure 4. Results obtained for orange drink samples spiked at 1.0 and 10.0 μg DSS/g. Alltech CN column. Conditions as described in text.

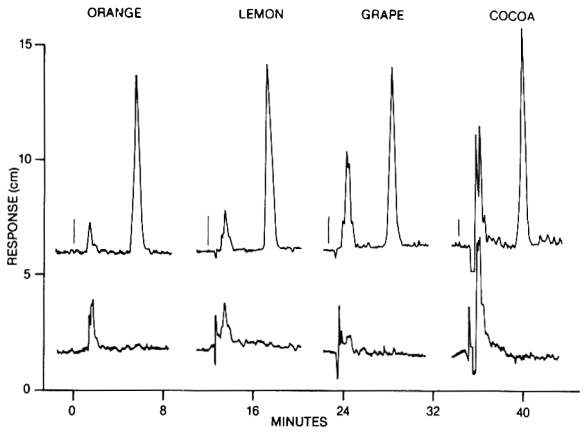

Figure 5. Chromatograms obtained for 4 beverage bases by direct extraction and analysis. Upper tracings are samples spiked at 10.0 μg DSS/g. Lower tracings are the respective unspiked samples. C-18 column. Conditions as described in text.

by a factor of about 6 compared with 657 nm, the sensitivity was more than adequate to detect DSS in the samples at the regulatory limit of 10 μg/g (15).

Chromatography

Figure 3 illustrates the separation of DSS from several sulfonic acids. All systems were satisfactory for DSS determinations. With the CN columns studied, hexadecane sulfonic acid always appeared as a broad peak. The reason for this was not investigated. We noted, however, that the compound was poorly soluble in mobile phases with 20–40% acetone while being more soluble in those containing 50–60% acetone. DSS has also been well separated from other alkane sulfonic acids by normal phase chromatography (9). In the present work, we found that the capacity factor for DSS was significantly affected by mobile phase composition for all of the systems studied. A few percent increase in acetone concentration decreased elution time by 10–20%. Increasing salt concentration in the mobile phase also caused an increase in retention time. These effects have been observed with other sulfonic acids when a C-18 column and a similar mobile phase (10) were used. With any given mobile phase preparation, the retention times were very reproducible, varying by less than ±1.0% for 4 replicate injections and less than ±2% over the course of a working day.

Sample Analysis

Figure 4 shows chromatographic results for spiked orange drink samples analyzed with a CN column as outlined in the experimental procedure after colorimetric analysis. No interferences were observed for DSS in any of the samples analyzed including fruit punch, cherry, grape, lemon, orange, and cocoa drinks. The results obtained after LC analysis compared exceptionally well with those obtained by the FDA colorimetric procedure on spiked samples of fruit-flavored drinks. For example, at the regulatory limit of 10 μg/g, the values obtained from duplicate spiked samples were 9.8 and 9.9 μg/g by colorimetry and 10.2 and 9.8 μg/g by liquid chromatography. This same correlation was obtained for DSS when carried through both analytical procedures at levels of 1.0, 2.0, 4.0, 6.0, and 8.0 μg/g. Results of the 2 methods differed by less than 2% over this range.

In a limited survey of 6 cocoa and 15 fruit-flavored beverage bases, no DSS was detected. In several of the chocolate-based samples, methylene blue-extractable substances were occasionally detected in the range 1–9 μg/g by the colorimetric procedure. However, LC analysis indicated that these results were not due to DSS.

In an effort to conduct rapid analyses for DSS in beverage bases, drinks were prepared using mobile phase, filtered, and then directly analyzed by liquid chromatography. Figure 5 shows some typical results obtained using a short C-18 column. As can be seen, the blank samples contained no DSS, although the cocoa appeared to contain other methylene blue complexing material. Recoveries at 10 μg/g were greater than 90% for the samples analyzed.

This method has the potential to be useful for the analysis of many substances which can combine with suitable dyes to form organic soluble ion pairs. The work described herein shows the utility of post-column, ion-pair extraction for substances that are otherwise difficult to determine. The selectivity of the detection system, as demonstrated in this work for direct beverage base analysis and for other biological samples (11, 12), should enable the method to be applied to other food matrixes and food additives.

Acknowledgment

The author thanks J. R. Iyengar and A. Telli for their technical assistance.

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Headspace Gas Chromatographic Determination of Residual 1,3-Butadiene in Rubber-Modified Plastics and Its Migration from Plastic Containers into Selected Foods

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A headspace gas chromatographic procedure has been developed for the determination of 1,3-butadiene in rubber-modified plastics and in some foods. Polymer solutions or foods are equilibrated in sealed vials at 90°C, and headspace samples are injected into a gas chromatograph. 1,3-Butadiene residues are measured using a flame ionization detector and are quantitated by the method of standard additions or an external calibration curve. Refrigerator tubs, vegetable oil bottles, chewing gum, and foods in contact with this type of packaging were analyzed. Limits of quantitation varied with the matrix, ranging from 2 ng/g (ppb) in chewing gum to 20 ng/g in polymers. 1,3-Butadiene was found in one polymer at 53 ng/g with an 8% coefficient of variation. The procedure yields "apparent" trace levels of 1,3-butadiene, and confirmation by a complementary technique is required.

Recent inhalation studies have shown that 1,3-butadiene (BD) is a potent carcinogen in rats and mice (1, 2). The effect of BD in the air of the workplace and the environment is of concern to some regulatory agencies (3). The U.S. Food and Drug Administration (FDA) is interested in knowing how much residual BD is present in BD-based polymers used in food packaging, chewing gum bases, and articles which come into contact with foods. When the BD levels in food-contact articles are known, FDA is able to determine if there is any potential human health risk.

Rubber-grade BD ranked among the top 50 chemicals produced in the United States in 1984 (4). BD is a major feedstock in manufacturing of synthetic rubbers and is easily homopolymerized or copolymerized with styrene and/or acrylonitrile. In addition, it can be grafted or blended with other polymers to form products exhibiting high mechanical resistance. Although BD polymers are widely used by industry, their use in food-packaging polymers is not common.

Most efforts to determine trace BD residues have been directed toward examining volatile organics in air instead of in polymeric materials and foods. The procedures for determining BD in air involve adsorbing volatile organics in large volumes of air in adsorption tubes, desorbing and concentrating the organics with solvent, and analyzing the extract for BD using gas chromatography (GC). These procedures are reliable and are accepted by both government and industry (5; The International Institute of Synthetic Rubber Producers, private communication, 1985).

Our approach to the determination of volatiles in polymers, foods, and other matrixes has been headspace sampling and GC (6–11). This technique uses an elevated sampling temperature, thereby enhancing volatilization into the partitioning vapor phase. Manual sampling of the vapor phase allows for large gaseous injections, and use of column backflush eliminates high-boiling solvent interferences, thus reducing analysis time. GC determination of BD residues in polymers and foods requires preconcentration, and manual headspace sampling is such a technique. Others have been successful in determining BD in margarine and plastic storage tubs with automated headspace sampling and mass spectrometric (MS) detection using single ion monitoring (12). This technique, although effective, is not routinely practiced in the analytical laboratory. An alternative preconcentration step uses purge-trap instrumentation, which has been used for concentrating BD residues and other volatiles in air samples before GC/MS confirmation (13), but the technique is difficult and time-consuming when quantitation is desired.

The GC column must resolve BD from the light gases; it also must retain and resolve BD from hydrocarbons of C-3 or less and still resolve BD from all C-4 unsaturated hydrocarbons.

This paper describes a procedure for the determination of BD in various matrixes, using manual headspace sampling and GC.

Experimental

Caution: BD is a teratogen and carcinogen. Use necessary safety measures when handling this compound.

Apparatus

(a) *Headspace sample vials.*—22 mL, with 12 × 20 mm finished tops and aluminum seals (Shamrock Glass Co., Linwood, PA 19061). Tuf-Bond Teflon-faced/silicon disks (No. 12720, Pierce Chemical Co., Rockford, IL 61105).

(b) *Syringes.*—10, 50, and 100 μL Hamilton 700 series and 500 μL Hamilton 1000 series; 5 mL Pressure-LOK, series A-2 gas syringes (Precision Sampling Corp., Baton Rouge, LA 70815).

(c) *Forced air oven.*—Stable-Therm constant temperature cabinet (Blue M Electric, Blue Island, IL 60406). Oven stabilized at 90°C.

(d) *Gas chromatograph.*—Perkin-Elmer Model 3920 with flame ionization detector. Operating conditions: temperatures (°C)—injection port 85, detector interface 150, column 75; gas flows (mL/min)—helium carrier 20, air 350, hydrogen 60; electrometer setting 2×10^{-12} amp full scale.

(e) *Chromatographic column.*—2 ft (61 cm) + 4 ft (122 cm) × ⅛ in. (3.2 mm) od coiled nickel SP-Alloy (T-1) backflush column (14), packed with 0.19% picric acid on 80–100 mesh Carbopack C (Supelco, Inc., Bellefonte, PA 16823).

(f) *Spectrum filter and amplifier.*—No. 1021A (Spectrum Scientific Corp., Newark, DE 19711). Noise filtering is used when amplifier setting is 1×10^{-12} amp full scale.

Reagents

(a) *Gases.—BD.*—3 lb (1.4 kg) cylinder (instrument grade); *cis-2-butene.*—3 lb (1.4 kg) cylinder (C.P.) (Matheson Gas Products, Dorsey, MD 21227); *trans-2-butene.*—150 g cylinder, ≥99.8% (GC) (Fluka AG, Buchs SG, Switzerland).

(b) *o-Dichlorobenzene (DCB).*—Distilled-in-Glass grade (Burdick & Jackson Laboratories, Inc., Muskegon, MI 49442). Demonstrate absence of materials coeluting at retention time of BD under conditions of headspace GC analysis. This laboratory sparges all high-boiling polymer solvents with helium at 70°C before use.

Received December 11, 1985. Accepted April 11, 1986.
Presented in part at the 36th Annual Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy, Feb. 25–March 1, 1985, New Orleans, LA.

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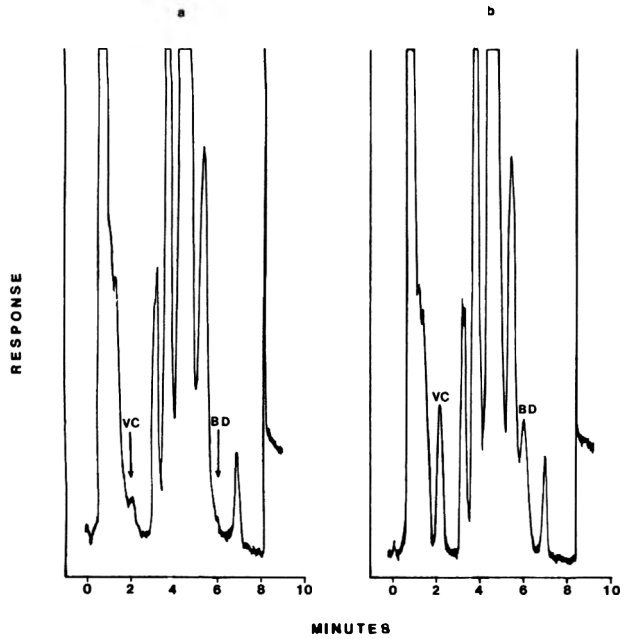

Figure 1. Chromatograms of 2 mL headspace samples from 10 mL 10% solution of BD rubber-modified PVC in DCB. Detector attenuation 1 × 1; cut-off frequency 0.1 Hz. a, unfortified sample, arrows indicate retention times for VC and BD; b, sample fortified with 50 ng VC and BD.

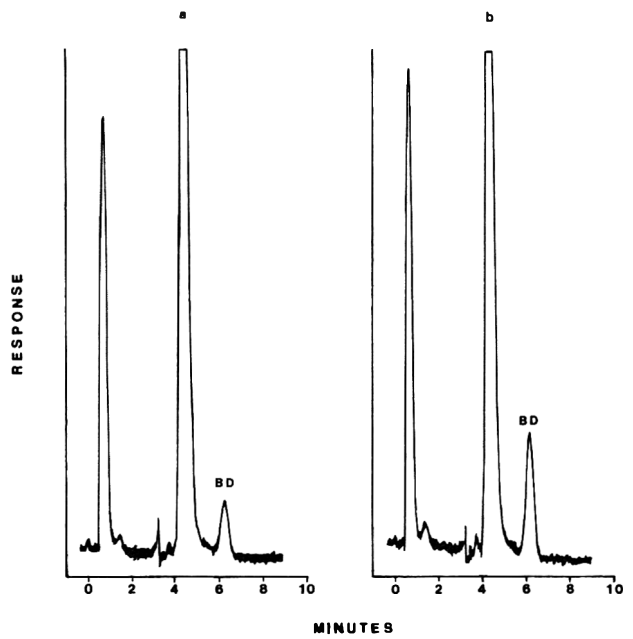

Figure 2. Chromatograms of 2 mL headspace samples from 10 mL 10% solution of 9.4% BD rubber-modified polystyrene resin in DCB. Detector attenuation 2 × 1; no signal filtering was used. a, unfortified sample; b, sample fortified with 60 ng BD.

(c) *Qualitative mixed gas standard.*—In fume hood, add ca 0.25 mL liquid from each inverted, freezer-cooled gas cylinder (a) to 3 separate headspace vials. Crimp-seal and label each vial. Transfer 10 μL aliquots from each vial into sealed, empty fourth vial by piercing septum and pumping syringe plunger 5 times at start and end of each transfer. Five μL injections of this final mixed vapor standard are used to check chromatographic column performance and optimum backflush start time. Mixed gas standard is prepared when needed.

(d) *BD standard solutions.*—(*1*) *Stock solution.*—About 20 000 μg BD/g DCB. Weigh empty headspace vial, cap, and septum to nearest 0.1 mg. Transfer 20 mL DCB to vial; reweigh with cap and septum. Place opened vial in fume hood and quickly add ca 0.4 g liquefied BD from inverted, freezer-cooled gas cylinder. Quickly cap and crimp-seal vial, let equilibrate 15 min, reweigh until constant weight is achieved, and calculate BD concentration. (*2*) *Intermediate solution.*—About 100 μg/mL. Transfer 20 mL DCB to headspace vial and weigh with cap and septum. Quickly add 75 μL stock solution, crimp-seal, reweigh vial, and calculate BD concentration. (*3*) *Spiking solution.*—About 1 μg/mL. Transfer 20 mL DCB to headspace vial, quickly add 200 μL intermediate solution, cap, seal, and calculate BD concentration.

Preparation of Headspace Standards and Samples

Polymers and liquid foods.—Cut plastic containers into ca 5 × 5 mm pieces. For 10% polymer solution, transfer 7.00 g sample to 125 mL Erlenmeyer flask containing 63 mL DCB and stirring bar. Cap and stir at room temperature until test portion dissolves, usually <4 h. Transfer 10 mL aliquots of polymer solution or liquid food to each of 5 headspace vials. Fortify vials 3, 4, and 5 with 1, 2, and 3 volumes, respectively, of BD spiking solution to give 2-, 3-, and 4-fold GC response for BD. Seal and shake vials. To remaining vials, add volume of DCB equivalent to volume of BD spiking solution added to vial 4 and seal.

Solid foods.—When possible, cut or mash food to ease transfer. Weigh 5.00 g food into each of 5 tared headspace vials. Proceed as described above for *Polymers and liquid foods.*

Determination Using Standard Additions

Place vial 1 of test series in forced draft oven. For liquids, semisolids, and polymer solutions, equilibrate 30 min. With heated syringe, pierce vial septum, draw full syringe of headspace vapor, expel volume into vial, and redraw 2 mL. Let syringe needle remain in vial headspace for ≥1 min in closed oven. Quickly close syringe valve, remove syringe, and inject 2 mL headspace into GC column. Under operating conditions described, BD elutes in ca 6 min. Remove vial 1 from oven, replace with next vial to be analyzed, wait 30 min, and repeat procedure.

Allow time (ca 3.5 min) for BD to pass 2 ft precolumn, then open backflush valve and backflush 20 min. Five vials can be analyzed in 3 h using this scheme.

Table 1. Apparent residual BD found in butadiene rubber-containing plastics

Product type	Apparent BD found, ng/g
9.4% S/SBR resin	54, 57, 48, 52
2.2% S/SBR resin	40
Olive oil bottle A	4600
Olive oil bottle B	5800, 6600, 5800
Vegetable oil bottle A	ND[a]
Vegetable oil bottle B	ND
Potato salad tub	77
Potato salad lid	1700
Cottage cheese tub	100
Yogurt tub lid	21
Chewing gum brand A	ND
B	ND
C	ND
D	ND
E	ND

[a] ND = not detected at ca 5 ng/g in vegetable oil and 0.5 ng/g in gum.

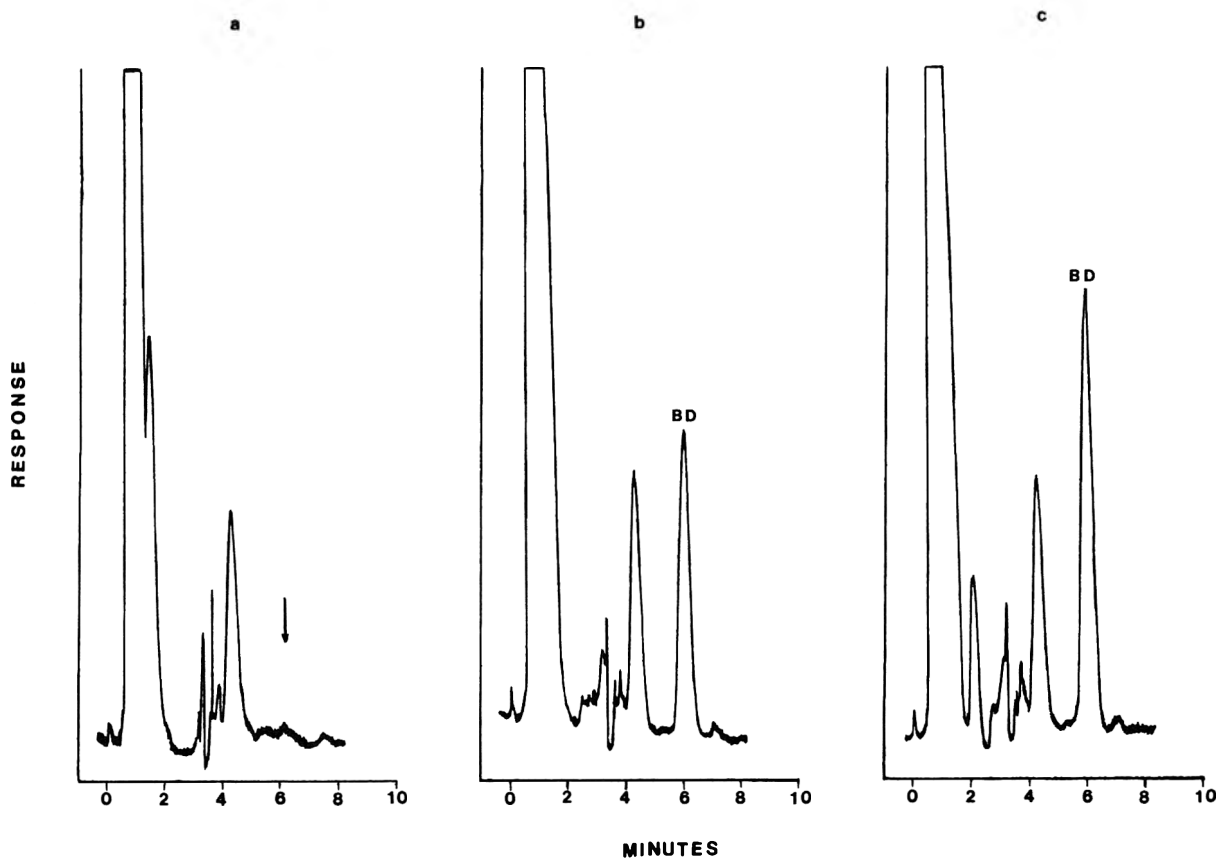

Figure 3. Chromatograms of 2 mL headspace samples from 10 mL olive oil. Detector attenuation 1 × 1; cut-off frequency 0.1 Hz. a, brand X olive oil bottled in glass; retention time of BD indicated by arrow; b, brand X olive oil bottled in plastic; c, brand X olive oil bottled in plastic fortified with 50 ng BD.

Calculation

Plot GC response for BD vs amount BD added for each vial analyzed. Generate linear regression curve for data and extrapolate curve through X-axis. This intercept represents amount BD in test portion.

Results and Discussion

Previous work in our laboratories (unpublished data, 1984) in conjunction with the American Society for Testing and Materials and the Society of Plastics Industries had shown that a GC column packed with 0.19% picric acid on Carbopack C resolved vinyl chloride (VC) from polymer and solvent interferences using headspace GC analysis. Supelco Bulletin 738B (15) discusses the use of this column to resolve C-4 unsaturated compounds. Our application of this column to the separation of C-4 hydrocarbons showed elution in order of increasing unsaturation, i.e., BD eluted after the 2-butenes, which eluted after the butanes. This elution pattern met our initial column requirements, but because of the high absorption characteristics of graphitized carbon, we used a backflush column system to hinder the potential breakthrough of late-eluting compounds from previous injections.

Table 2. Migrating residual BD found in foods

Food	Apparent BD found, ng/g
Olive oil A	8, 8
Olive oil B	8
Olive oil C	8, 9
Vegetable oil A	ND[a]
Vegetable oil B	ND
Yogurt A	ND
Yogurt B	ND

[a] ND = not detected at ca 1 ppb.

Repeated injections of headspace samples, coupled with a high column inlet pressure, required septum replacement at the injection port after the analysis of each series of 5 or 6 headspace injections. Teflon-faced silicon septa were used for the injection port and headspace vials to prevent hydrocarbon contamination of the GC column and the test portion, if ordinary butyl septa were used.

DCB dissolved all the BD rubber-modified plastics analyzed except rubber-modified polyvinylchloride (PVC). At room temperature, PVC was only slightly soluble in DCB. Later, it was learned that each vial of the PVC sample series, when prepared independently (1 g test portion plus 9 mL DCB) and equilibrated at 90°C overnight, formed a gel. Headspace over the gel was then sampled and the series was analyzed by GC. Figure 1 shows chromatograms representative of the analysis for residual BD in fortified and nonfortified rubber-modified PVC. No apparent BD was found in the plastic. Chromatographic column resolution allows simultaneous analysis for BD and VC in the rubber-modified PVC. No residual VC was found in the plastic. The responses observed for VC and BD in Figure 1b are about 40 and 30 mm, respectively.

Figure 2 shows the GC results of the analysis of a 9.4% BD rubber-modified polystyrene resin. The response observed for BD in Figure 2a was 16 mm. To demonstrate the method's precision, the same 9.4% BD rubber-modified polystyrene was analyzed 4 times. Apparent residual BD was 53 ng/g with a coefficient of variation of 8%. Table 1 lists the BD rubber-modified plastics analyzed and apparent BD found, using the technique described. The lower limit of

quantitation for BD in the products analyzed was 20 ng/g at 1×10^{-12} amp full scale. This BD response was 5 times greater than baseline noise.

Five different brands of chewing gum with BD rubber bases were analyzed using solid-vapor phase partitioning. Dissolution of the gum was not attempted. Each test portion was equilibrated 1 h at 90°C before analysis. No apparent BD was found in any of the products, and good linear correlation was achieved when the BD response level of fortification was plotted. Using the method of standard additions, the limit of quantitation was 2 ppb in chewing gum.

Olive oil, bottled in BD rubber-modified acrylonitrile–acrylic bottles, was analyzed for residual BD. BD residues were determined using an external calibration curve and the method of standard additions. Figure 3 shows chromatograms from olive oil analysis. The response for BD in Figure 3b, the unfortified sample, was 66 mm. Apparent BD found in the sample was 8 ng/mL.

Table 2 summarizes the analyses for apparent BD in various foods packaged in BD rubber-based plastics. The vegetable oil was packaged in rubber-modified PVC. Yogurt tubs were polystyrene with rubber-modified polystyrene lids. Five baby bottle nipples from 3 sources were screened for BD rubber content using infrared (IR) spectrophotometry. None of the spectra exhibited any characteristic absorption bands of BD rubbers; therefore, determination of residual BD was not attempted.

A procedure has been described for the determination of BD residues in a number of matrixes. The method of standard additions was used to quantitate apparent BD levels in all of the products analyzed. The residue measured, using the method of standard additions, represents the maximum level present until confirmed by another procedure such as GC/MS or IR spectrophotometry. Applying this method, apparent residual BD was found in food packaging at levels ranging from 21 ng/g to 6600 ng/g. Of all the foods packaged in polybutadiene-based plastics, only olive oil contained measurable BD residues.

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SAMPLES AND SAMPLE HANDLING

Effects of Freezing on Nitrite Stability in Aqueous Solutions

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The effects of freezing on nitrite stability in microbial cultures and in other liquid media were examined. Nitrite (12-13 mg $(NO_2^-)N$/100 mL, pH 5.7) in culture and aqueous media was very unstable during frozen storage. Freezing resulted in the degradation of nitrite to other products including nitrate. Losses of 38-57% of the nitrite occurred after 15 days of frozen storage. Neither addition of chloroform nor a 5 min boiling treatment before freezing prevented nitrite destruction. However, nitrite concentration in the unfrozen portion of aqueous media increased during the freezing process. Nitrite can be stabilized during frozen storage by increasing the pH to near 11.0. For aqueous solutions, nitrite can be preserved by the use of chloroform or by increasing the pH to 11.0 and storing at 5°C.

Nitrate and nitrite are of major importance to agriculture and the food industry. Nitrate is a valuable source of nitrogen for plants; however, bacterial denitrification can result in large losses of nitrogen from soils. The so-called silo filler's disease in livestock arises from bacterial nitrate reduction to nitrite in silage. Excessive levels of nitrate in drinking water (from ground wells), in some leafy and root vegetables, and in livestock feed can cause methemoglobinemia in infants and livestock from the reaction of hemoglobin with nitrite formed by microbial nitrate reduction. At one time, formation of carcinogenic nitrosamines from the reaction of nitrite with secondary amines was considered a major problem in cured meats. Now, careful control of nitrite addition in the curing process has minimized nitrosamine formation. Good reviews on the importance of nitrates and nitrites in food and agriculture are available (1, 2).

In any study of nitrite, the preservation of extracts for later analyses may be necessary. Preservation of the sample to prevent loss of nitrite due to microbial growth or chemical reactions is necessary. Freezing is often the preferred method for storing samples. Chloroform can be added to inhibit microbial growth in aqueous extracts. Care must be taken with nitrite because it is chemically unstable at acid pH but stable at basic pH. Our studies on the variability of the nitrate and nitrite content of bacterial cultures undergoing nitrate reduction to nitrite showed that stability problems existed in certain frozen samples containing nitrite. This study examines the effects of freezing, chloroform plus freezing, and pH on nitrite stability. A method to stabilize nitrite during freezing is suggested.

Experimental

A nitrate-reducing bacterium isolated from corned beef and pastrami and tentatively identified as *Paracoccus denitrificans* was used in this study.

The medium of Spangler and Gilmour (3) was modified to contain the following (g/L): yeast extract, 1.0; $(NH_4)_2HPO_4$, 0.5; KH_2PO_4, 1.14; K_2HPO_4, 1.45; KNO_3, 1.0; $MgSO_4 \cdot 7H_2O$, 0.1; MoO_3, 0.003; $FeSO_4$, 0.001; glucose, 5.00; and water to 1 L. The glucose and $MgSO_4 \cdot 7H_2O$ were sterilized together and added after autoclaving to prevent precipitation of the magnesium. One mL of a 24 h culture grown in nutrient broth at 30°C was added to 100 mL sterile medium in each of several 250 mL Erlenmeyer flasks. The inoculated flasks were anaerobically incubated at 10° ± 0.5°C for 3 days. Anaerobiosis was obtained by placing the inoculated flasks in large vacuum desiccators, evacuating to 50–55 mm Hg, and introducing helium to atmospheric pressure. The flushing was repeated 4 times, and a final helium pressure of about 25 mm below atmospheric pressure was used.

Cultures were sampled after 3 days of incubation and pH was determined. The nitrite content attained a maximum of 12–13 mg/100 mL after 3 days of incubation. Twenty mL aliquots from the 3-day cultures were treated in the following ways:

Treatment	*Procedure*
None	water to 200 mL
KOH	water to 100 mL; 10% KOH to pH 11.0–11.2; water to 200 mL
Chloroform	water to 200 mL + 2 mL chloroform
Boiling	water to 100 mL; boiled 5 min; cooled; water to 200 mL.

The diluted cultures were divided into equal volumes and stored as required at either 5 or −20°C in 120 mL polypropylene bottles. The samples were analyzed after 1, 8, and 15 days of storage. The frozen samples were thawed in warm water to room temperature, and aliquots were diluted for the nitrate and nitrite analyses. Uninoculated controls were treated in the same manner as the sample cultures.

For the freezing experiments with uninoculated media, KNO_3 was eliminated and $NaNO_2$ was added to obtain $(NO_2^-)N$ levels similar to those found in the inoculated samples. The experiment with media minus the organic matter eliminated the yeast extract and glucose.

The fractional freezing experiments were conducted by weighing 200 g solution into preweighed 250 mL Erlenmeyer flasks. The flasks were placed in a freezer at −20°C. Flasks were removed when approximately ¼, ½, and ¾ of the liquid was frozen. The liquid phase was poured into another preweighed flask for weight determination. The weight of the ice was determined by difference. The ice was thawed and the samples were adjusted to pH 11.0 and frozen for later analyses.

Nitrate and nitrite analyses were performed on a Techni-

Table 1. Effect of freezing storage on nitrite in *P. denitrificans* culture (pH 5.7)

Treatment before freezing	$(NO_2^-)N$ content, mg/100 mL[a]		
	Days in frozen storage		
	1	8	15
None	11.1	7.3	6.7
Chloroform	11.2	8.5	7.7
Five-min boiling	11.1	7.3	5.4
pH 11.0	12.5	12.6	12.5

[a] Average of 3 samples.

Received May 9, 1985. Accepted April 15, 1986.
Published with the approval of the Director of the Idaho Agricultural Experiment Station as Research Paper No. 8158.

Table 2. Changes in $(NO_2^-)N$ and $(NO_3^-)N$ in frozen *P. denitrificans* culture (pH 5.7)

Days in frozen storage	mg N/100 mL[a]	
	$(NO_3^-)N$	$(NO_2^-)N$
1	1.4	11.1
8	3.4	7.3
15	3.7	6.7

[a] Average of 3 samples.

con AutoAnalyzer I using the Kamphake et al. method (4). Nitrate required reduction to nitrite with alkaline hydrazine sulfate before the colorimetric determination with sulfanilamide and *N*-1-naphthylethylenediamine dihydrochloride. Results of nitrite analyses by the AutoAnalyzer I and by the APHA colorimetric procedure (5) were the same.

Results and Discussion

Table 1 shows the effect of freezing plus storage on the $(NO_2^-)N$ of diluted 3-day cultures. Comparisons should be made with the pH 11.0 treatment because the nitrite is stable at this pH (see later discussion). The samples with chloroform, 5-min boiling, and no treatment showed losses of $(NO_2^-)N$ ranging from 32 to 42% in 8 days of frozen storage. Even 1 day of storage resulted in losses greater than 10%. The loss of $(NO_2^-)N$ after 15 days of storage ranged from 38 to 57%. The loss of $(NO_2^-)N$ during the second week of storage was much less than the first week. The presence of chloroform and the 5-min boiling treatment plus the freezing temperature indicate that neither microbial growth nor enzymes are involved in the loss of nitrite.

Table 2 shows that some of the nitrite is converted to nitrate. Nitrate formation occurs mainly during the first week and decreases considerably during the second week.

The possibility that the instability of the nitrite was due to a pH effect was tested by storing the solutions as a liquid at 5°C. Solutions at pH 11.0 tested at 12.5, 12.3, and 12.6 mg/100 mL at 0, 7, and 15 days; solutions with chloroform tested at 12.5, 12.3, and 12.6 mg/100 mL at 0, 7, and 15 days. Thus, the large $(NO_2^-)N$ changes occurring during freezing are not due to pH effects but are the result of a combination of pH and freezing effects.

Another possible cause of the loss of $(NO_2^-)N$ could be related to the culture composition effects. This was tested by adding nitrite to uninoculated media. Table 3 shows that nitrite is lost even in the absence of bacteria. Therefore, the culture composition was not responsible for the large $(NO_2^-)N$ loss. Table 3 also shows the greater stabilization of the nitrite as the pH increases to 6.8. A pH of 11.0 was chosen for sample preparation because this pH stabilizes nitrite and would be a good inhibitor of microbial growth.

The possible reaction of nitrite with organic constituents such as the amino acids was tested by eliminating the organic constituents from the media. Solutions at pH 5.7 without organic matter tested at 10.8, 7.4, and 6.4 mg/100 mL at 1, 8, and 15 days of frozen storage; solutions at pH 11.0 showed 14.2, 14.3, and 14.3 mg/100 mL at 1, 8, and 15 days of storage. The loss of nitrite indicates that reaction with organic constituents was not a significant factor for nitrite loss in the frozen samples.

Table 3. Effect of pH and frozen storage on nitrite stability in uninoculated media

pH of media	$(NO_2^-)N$, mg/100 mL[a]			
	Days of frozen storage			
	0	1	8	15
5.7	14.4	9.7	4.3	4.4
6.2	14.5	11.9	7.7	6.5
6.8	14.4	13.9	11.0	11.1
11.0	14.5	14.5	14.4	14.5

[a] Average of 3 samples.

Table 4. Concentration of nitrite in ice and liquid phases of freezing media (pH 5.7)

Phases	Time in freezer	Wt of ice or water, g	$(NO_2^-)N$/100 mL[a]
¼ frozen	1 h 10 min to 2 h	liquid: 154–160	13.6
		ice: 40–46	7.6
½ frozen	1 h 10 min to 3 h 10 min	liquid: 94–110	18.2
		ice: 90–106	7.3
¾ frozen	4 h to 4 h 20 min	liquid: 35–64	27.0
		ice: 136–165	7.8
Unfrozen control (pH 11.0)			12.2

[a] Average of 4 samples.

The results of the previous experiments indicate the large changes in $(NO_2^-)N$ were caused by the freezing process. An analogous enhanced reaction due to freezing has been observed in the more rapid autooxidation of lipids in frozen herring (6). The accumulation of solutes in the liquid phase during freezing may be a major reason why unexpected reactions can occur. Table 4 shows the changes in $(NO_2^-)N$ in the liquid and ice phases as the solutions freeze, the most obvious being the increases in $(NO_2^-)N$ in the liquid phase as the solution freezes. When the solutions are ¾ frozen, the $(NO_2^-)N$ in the liquid phase is more than twice that of the original solution. The progressive increase in $(NO_2^-)N$ in the liquid phase could result in unexpected loss of $(NO_2^-)N$ during freezing. The results of this freezing test varied much more than the previous experiments. Wide differences in time to obtain a specific freezing fraction probably account for much of the differences. For example, the time to obtain solutions which were ¾ frozen varied from 4 to over 6 h. The many fractional freezing experiments which were performed indicated that the freezing process and the accumulation of solutes $(NO_2^-)N$ was complicated.

The concentration of $(NO_2^-)N$ in the ice phase was similar regardless of the fraction of the sample which was frozen. This similarity was also found in other fractional freezing experiments.

The loss of nitrite when samples are frozen at pH 6.8 or lower emphasizes the importance of the sample preservation method in nitrate and nitrite analyses. The pH of the solutions to be analyzed should be near pH 11.0 for either frozen or refrigerated storage. Chloroform was effective for preserving nitrite at pH 5.7 and 5°C but not in frozen storage.

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METALS

Rapid Determination of Methyl Mercury in Fish and Shellfish: Method Development

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The AOAC official first action method for methyl mercury in fish and shellfish was modified to provide more rapid determination. Methyl mercury is isolated from homogenized, acetone-washed tissue by addition of HCl and extraction by toluene of the methyl mercuric chloride produced. The extract is analyzed by electron capture gas chromatography (GC) on 5% DEGS-PS treated with mercuric chloride solution. The quantitation limit of the method is 0.25 μg Hg/g. Swordfish, shark, tuna, shrimp, clams, oysters, and NBS Research Material-50 (tuna) were analyzed for methyl mercury by the AOAC official first action method. All products also were analyzed by the modified method and the AOAC official method for total Hg. In addition, selected extracts obtained with the modified method were analyzed by GC with Hg-selective, microwave-induced helium plasma detection. There was no significant difference between the results for the various methods. Essentially all the Hg present (determined as total Hg) was in the organic form. Coefficients of variation from analyses by the modified method ranged from 1 to 7% for fish and shellfish containing methyl mercury at levels of 0.50-2.30 μg Hg/g. The overall average recovery was 100.5%.

The U.S. Food and Drug Administration (FDA) regularly monitors fish and shellfish for Hg and recommends that regulatory action be taken when levels are greater than 1.0 μg Hg/g. This action level, which was established in 1978 (1), was derived from data on human consumption of fish and toxicity of methyl mercury. From 1978 to 1984, enforcement of the 1.0 μg Hg/g action level was based on determination of total Hg (2), although methyl mercury is the most toxic form of this element present in human food (3). There were 2 reasons for monitoring total Hg instead of methyl mercury: Almost all of the mercury in most fish species is present in the methylated form (4), and an official method for methyl mercury was not yet available. To meet the need for such an official method, FDA developed a gas chromatographic (GC) procedure which was collaboratively studied (5) and adopted official first action by AOAC in 1983 (6). In 1984, it was then possible for FDA to change the basis of enforcement of the 1.0 μg Hg/g action level from total Hg to Hg in the form of methyl mercury (7).

The GC method adopted by AOAC in 1983 has several disadvantages: The use of benzene is undesirable for safety reasons; evaporative concentration and quantitative transfers are time consuming and introduce potential for dilution errors; and the electron capture (EC) detector of the gas chromatograph may respond to other compounds in addition to methyl mercuric chloride if the tissue is not thoroughly preextracted to remove interferences. The purpose of this work was to address these problems.

In the modified EC-GC method presented here, we substituted the use of toluene for benzene and eliminated the evaporative concentration step. Results for the modified method are equivalent to those for the AOAC official first action methods for methyl mercury and total Hg. We subsequently initiated a collaborative study of the modified method; the results of the study will be the subject of a future report.

In addition to comparing the results for the modified EC-GC method with those for AOAC official methods, we investigated and validated the adequacy of sample cleanup for EC detection by monitoring the GC effluent with an Hg-specific, microwave-induced helium plasma (MIP) detector. MIP-GC detection of methyl mercuric chloride in fish was reported by Bache and Lisk (8), who used the Westöö method (9). In this work, we used the modified method with MIP detection to confirm the presence of Hg in selected extracts and to quantify the Hg at the methyl mercuric chloride retention time. Our results obtained by MIP-GC demonstrate that the modified EC-GC method produces no coeluting interferences.

Received December 19, 1985. Accepted May 28, 1986.

Experimental

Product Selection

Three varieties each of fish and shellfish were selected for study. Swordfish and shark were chosen because they frequently contain high levels of Hg. Shrimp, clams, oysters, and canned tuna were chosen to represent commonly consumed seafoods.

Sample Preparation

Fresh swordfish, shark, shrimp, and oysters, and canned clams and tuna (water-packed) were purchased locally. The shrimp were peeled and the tuna and clams were drained before homogenization. Approximately 300 g of each accurately weighed seafood was homogenized about 7 min (until the seafood appeared to be a uniform paste) in a food processor with a stainless steel blade. The shark was difficult to homogenize and required the addition of water (equal to 20% of tissue weight) to obtain a uniform paste. It was not necessary to add water to the swordfish, tuna, shrimp, oyster, and clam homogenates. Separately homogenized fresh frozen swordfish steaks (with various Hg levels) were used for the analytical parameter optimization experiments.

For recovery experiments, swordfish A, shark, tuna A, clams, and shrimp were homogenized, weighed, individually fortified with a known amount of methyl mercuric chloride, refrigerated at 8°C, and analyzed within 24 h after homogenization. Swordfish B, swordfish C, tuna B, tuna C, and oysters were homogenized, fortified, frozen in bulk, and then thawed and weighed immediately before analysis.

Total Mercury—AOAC Method

Total Hg was determined by the AOAC official alternative method for fish (2) by solubilizing the test portion with a sulfuric acid–nitric acid mixture and V_2O_5. The digestion was carried out exactly as described in the procedure. The partial digest was analyzed for Hg by the method of standard addition to ensure that undigested fat did not cause erroneous results. A Perkin-Elmer Model 403 atomic absorption spectrophotometer equipped with an Hg hollow cathode lamp, deuterium background correction, and an 11.5 cm, closed end, quartz, flow-through cell was used for analysis.

Methyl Mercury—AOAC Method

Methyl mercury was determined by the AOAC official first action method (5, 6). To conserve reagents, the extraction

was carried out with the following modifications: A 1 g portion of homogenized tissue was prewashed 3 times with 10 mL portions of acetone and once with 10 mL benzene. The prewashed tissue was acidified with 5 mL HCl–water (1 + 1) and extracted 3 times with 10 mL portions of benzene. The phases were separated by centrifugation at 20°C. The combined benzene extracts were concentrated in Kuderna-Danish glassware equipped with a new style of Snyder column (No. K-503100-003, 170 mm long, 2 bulbs, Kontes Co., Vineland, NJ 08360). The extracts were diluted to 25 mL with benzene, mixed with 5 g Na_2SO_4, and analyzed by EC-GC as described in *Apparatus* (g).

MIP Detection

To provide Hg-specific methyl mercuric chloride detection, the gas chromatograph was interfaced to the MIP system described by Carnahan (10). The GC column described in *Apparatus* (g) was interfaced to the MIP detection system with a manual switching valve to allow solvent venting. The column was switched to the MIP system 1–1.5 min after injection for methyl mercuric chloride detection at ca 3 min. Helium gas flow was 30 mL/min at all times. The MIP system consisted of a 2450 MHz microwave generator (Opthos Instruments, Rockville, MD) and a TM_{010} microwave cavity (Beenakker design) fitted with a 2–3 mm id × 6–8 mm od quartz discharge tube. Power was maintained at 75–80 watts. The cavity was tuned with a 3 stub tuner. Light emission was measured at 253.45 nm by using a Jarrell-Ash scanning monochromator (Model 82-000, Waltham, MA 02254) with 3 mm × 50 μm entrance and exit slits and a type R212UH photomultiplier tube (Hamamatsu Corp., Middlesex, NJ 08846). A mercury lamp was used to manually select the wavelength of maximum emission. Maintenance of wavelength for maximum emission was verified with the Hg lamp throughout the day to check for drift. The photomultiplier tube was powered by a Model 240A high voltage supply (Keithley Instruments, Cleveland, OH 44139). The signal was processed by a Model 1021 filter and amplifier (Spectrum Scientific Corp., Newark, DE 19711) and Model 417K chromatograph electrometer (Keithley Instruments). Output was recorded on a Hewlett-Packard 7131A recorder.

Methyl Mercury—Modified Method

Methyl mercury was also determined by the following modified EC-GC method, which is the subject of this report.

METHOD

Principle

Organic interferences are removed from homogenized seafood by an acetone wash followed by a toluene wash. Protein-bound methyl mercury is released by the addition of HCl and is extracted into toluene. The toluene extract is analyzed for methyl mercuric chloride by EC-GC.

Reagents

(a) *Solvents.*—Acetone, toluene, and isopropanol, all distilled in glass (Burdick & Jackson Laboratories Inc., Muskegon, MI 49442, or MC/B Manufacturing Chemists, Norwood, OH 45212). *Warning:* Toluene is harmful if inhaled and is flammable; conduct all operations with toluene in laboratory hood.

(b) *Hydrochloric acid solution (1 + 1).*—Add concentrated HCl to equal volume of distilled or deionized water and mix. Use 2 volumes of toluene to extract potential interferences from 1 volume of HCl solution by vigorously shaking mixture in separatory funnel for 15 s. Discard toluene extract. Repeat extraction step 4 times. Solution may be mixed in advance. However, the extraction must be performed immediately before HCl solution is used to avoid formation of electron-capturing compounds which produce extraneous peaks in chromatograms.

(c) *Carrier gas.*—GC quality argon–methane (95 + 5).

(d) *Sodium sulfate.*—Heat overnight in 600°C furnace, let cool, and store in capped bottle. Line cap with acetone-washed aluminum foil to prevent contamination from cap.

(e) *Methyl mercuric chloride standard solutions.*—Keep tightly stoppered. Seal stopper with Teflon tape. *(1) Stock solution.*—1000 μg Hg/mL. Weigh 0.1252 g methyl mercuric chloride (ICN-K&K Laboratories Inc., Plainview, NY 11803) into 100 mL volumetric flask. Dilute to volume with toluene. *(2) High level intermediate solution.*—40 μg Hg/mL. Dilute 10.0 mL stock solution to 250.0 mL with toluene. *(3) Low level intermediate solution.*—2.0 μg Hg/mL. Dilute 10.0 mL high level intermediate solution to 200.0 mL with toluene. *(4) Working standard solutions.*—0.005–0.10 μg Hg/mL. Prepare monthly by diluting with toluene in volumetric flasks as follows: Dilute 10.0 mL of 2.0 μg Hg/mL solution to 200.0 mL for 0.10 μg Hg/mL. Dilute 20.0 mL of 0.10 μg Hg/mL solution to 25.0 mL, 15.0 mL to 25.0 mL, 10.0 mL to 25.0 mL, 10.0 mL to 50.0 mL, 10.0 mL to 100.0 mL, and 10.0 mL to 200.0 mL for 0.080, 0.060, 0.040, 0.020, 0.010, and 0.005 μg Hg/mL, respectively.

(f) *Mercuric chloride column treatment solution.*—1000 ppm mercuric chloride. Dissolve 0.1 g mercuric chloride in 100 mL toluene.

(g) *Fortification solutions.*—*(1) Stock solution.*—1000 μg Hg/mL. Weigh 0.1252 g methyl mercuric chloride into 100 mL volumetric flask. Dilute to volume with water. *(2) Working fortification solution.*—15 μg Hg/mL. Dilute 1500 μL stock fortification solution to 100.0 mL with water.

Apparatus

Wash all glassware with detergent (Micro Laboratory Cleaner, International Products, Trenton, NJ 08601) and rinse thoroughly with hot tap water followed by distilled or deionized water. Then rinse 3 times with acetone and 3 times with toluene. Dry in hood.

(a) *Centrifuge.*—Model IEC CRU-5000 (International Equipment Co., Needham Heights, MA 02194).

(b) *Centrifuge tubes.*—50 mL with Teflon-lined caps (Cat. No. 9212-K78, Arthur H. Thomas Co., Philadelphia, PA 19105).

(c) *Graduated cylinders.*—Class A, 50 mL, with ground glass stoppers (No. 20036, Kimble Div., Owens-Illinois, Inc., Toledo, OH 43666).

(d) *Transfer pipets.*—Disposable glass, Pasteur-type.

(e) *Dropping pipets.*—5 mL (No. 13-710B, Fisher Scientific Co., Pittsburgh, PA 15219).

(f) *Mechanical shaker.*—Model S-500 shaker-in-the-round, with Model PT-0 timer (Kraft Apparatus, Inc., Mineola, NY 11501).

(g) *Gas chromatograph.*—Hewlett-Packard Model 5710A equipped with linear ^{63}Ni EC detector, Model 7131A recorder, and 6 ft × 2 mm id silanized glass column packed with 5% DEGS-PS on 100–120 mesh Supelcoport (Supelco, Inc., Bellefonte, PA 16823). Pack column *no closer* than 2.0 cm from injection and detector port nuts and hold packing in place with 2 cm high quality, silanized glass wool at both ends. Install oxygen scrubber and molecular sieve dryer (No. HGC-145, Analabs, North Haven, CT 06473) between car-

rier gas supply and column. Condition column according to manufacturer's instructions as follows: Flush column 0.5 h with carrier gas at 30 mL/min at room temperature. Then heat 1 h at 50°C. Next, heat column to 200°C at 4°/min and hold at 200°C overnight. Do not connect column to detector during this conditioning process. Maintain 30 mL/min carrier gas flow at all times during conditioning, treatment, and use. Operating conditions: temperatures (°C)—column 155, injector 200, detector 300; carrier gas flow 30 mL/min; recorder chart speed 0.5–1.0 cm/min. Under these conditions and with mercuric chloride column treatment procedure described below, methyl mercuric chloride peak appears 2–3 min after injection of extract.

Mercuric Chloride Column Treatment

Five percent DEGS-PS, conditioned according to manufacturer's instructions, can be used to determine methyl mercuric chloride only after treatment by mercuric chloride solution (**f**). Because column performance degrades with time, also treat column periodically during use. Perform appropriate mercuric chloride treatment procedures described below.

(**a**) *Following 200°C column conditioning and after every 2–3 days of analyses.*—If column has just been conditioned according to manufacturer's instructions or has been used 2–3 days to analyze extracts, proceed as follows: Adjust column temperature to 200°C and inject 20 μL mercuric chloride treatment solution 5 times at 5–10 min intervals. Maintain 200°C temperature overnight. Chromatogram will contain large, broad peaks. Adjust column temperature to 155°C next morning and inject 20 μL mercuric chloride treatment solution 2 more times. Large, broad chromatographic peaks appearing at ca 1–2 h signal completion of treatment process and that column is ready for use.

(**b**) *On day preceding analyses.*—If column has been treated by procedure (**a**) or used 1 day at 155°C to analyze extracts, column may be treated at end of working day for next day's use as follows: Lower column temperature to 115°C and inject 20 μL mercuric chloride treatment solution one time. After large, broad peaks appear in chromatogram (11–20 h), treatment process is complete. Next working day, increase column temperature to 155°C operating temperature. When baseline is steady, column is ready for use.

(c) *During extract analyses at 155°C.*—If column has been used at 155°C to analyze extracts and if column performance has degraded enough to require mercuric chloride treatment, inject two 20 μL aliquots of mercuric chloride treatment solution. Large, broad peaks will appear in chromatogram 1–2 h after mercuric chloride injection, signaling completion of treatment process. Wait for steady baseline; then column is ready for use.

Extraction of Methyl Mercuric Chloride

Perform all operations except weighing in laboratory hood. Take empty centrifuge tube through all steps for method blank determination. Accurately weigh 1 g homogenized test sample into 50 mL centrifuge tube. Add 25 mL acetone; tightly cap and *vigorously* shake tube by hand 15 s. Loosen cap and centrifuge 5 min at 2000 rpm. Carefully decant and discard acetone. (Use dropping pipet to remove acetone, if necessary.) Repeat 25 mL acetone wash step 2 more times. Break up tissue with glass stirring rod before shaking tube, if necessary. Add 20 mL toluene; tightly cap and vigorously shake tube by hand 30 s. Loosen cap and centrifuge 5 min at 2000 rpm. Carefully decant (or draw off with dropping pipet) and discard toluene. Extraneous peaks in final GC chromatogram may indicate that more vigorous shaking with acetone and toluene is required. In products for which methyl mercury recoveries are to be determined, fortify tissue at this point by adding working fortification solution (**g**) to centrifuge tubes.

Add 2.5 mL HCl solution (**b**) to centrifuge tube containing acetone- and toluene-washed sample. Break up tissue with glass stirring rod, if necessary. Extract methyl mercuric chloride by adding 20 mL toluene and shaking tube *gently* but *thoroughly* 5 min on mechanical shaker at setting 5 (2 min by hand). Loosen cap and centrifuge 5 min at 2000 rpm. If emulsion is present after centrifugation, add 1 mL isopropanol to reduce emulsion. Gently stir isopropanol into toluene with glass stirring rod. Do not mix isopropanol with aqueous phase. Add equal amounts of isopropanol to blank and test solutions. If emulsion is not present, do not add isopropanol to blank or test solutions. Vigorous mixing of isopropanol with HCl may produce interfering peaks in chromatograms. Recentrifuge. With dropping pipet, carefully transfer toluene to graduated cylinder. Rinse walls of centrifuge tube with 1–2 mL toluene and transfer rinse to graduated cylinder. Repeat extraction step one more time. Combine both extracts in graduated cylinder, dilute to 50 mL with toluene, stopper, and mix well. Add 10 g Na_2SO_4 and mix again. Tightly stoppered extracts (sealed with Teflon tape) may be refrigerated and held overnight at this point. Analyze by GC.

Gas Chromatography

Verify that system is operating properly by injecting 5 μL standard solution containing 0.005 μg Hg/mL into gas chromatograph. The difference between methyl mercuric chloride peak heights for 2 injections should be ≤4%. Check linearity by chromatographing all working standard solutions.

Inject 5 μL standard solution with concentration approximately equal to or slightly greater than concentration of extract. Immediately after methyl mercuric chloride peak appears, inject 5 μL extract. Immediately after methyl mercuric chloride and background peaks for extract appear, inject another 5 μL aliquot of standard solution. Because column performance and peak height slowly decrease with time, calculate Hg concentration in each test sample by comparing peak height for each test extract to average peak height for standard solutions injected immediately before and after test extract.

Correct height of methyl mercuric chloride peak for test extract by subtracting height of peak for method blank obtained at same attenuation and recorder sensitivity. Calculate methyl-bound Hg content of test sample expressed as μg Hg/g (ppm Hg) by comparing height of peak from injection of test extract to average height of peak from duplicate injections of standard solution as follows:

$$\mu g\ Hg/g\ fish = (R/R') \times (C'/C) \times 50\ mL$$

where R = corrected height of methyl mercuric chloride peak from injection of test extract, R' = average height of methyl mercuric chloride peak from duplicate injections of standard solution, C = weight (g) of test portion, and C' = concentration (μg/mL) of Hg in standard solution.

Results

Table 1 presents the Hg levels in selected fish and shellfish, determined by the AOAC official method for methyl mercury (5, 6), the modified method for methyl mercury that was developed in this study, and the AOAC official method for

Table 1. Comparison of methods for determining Hg in seafood

Commodity	Hg, μg/g: Methyl mercury by AOAC method (3)	Methyl mercury by modified method	Total Hg by AOAC method (2)
Swordfish A	1.27	1.40	1.26
	1.24	1.33	1.41
	1.25	1.26	
	1.25	1.31	
		1.35	
Shark	1.88	2.16	2.11
	2.10	2.17	1.75
		2.07	
		2.18	
Tuna A	0.47	0.58	0.48
	0.49	0.55	
		0.55	
		0.52	
Clams	ND[a]	ND	ND
	ND	ND	
		ND	
		ND	
Shrimp	ND	ND	ND
	ND	ND	
		ND	
		ND	
Tuna[b]	1.00	0.98	0.88
	0.97	1.08	

[a] ND indicates not present above quantitation limit, i.e., 0.05 μg Hg/g for AOAC methyl mercury method, 0.25 μg Hg/g for modified methyl mercury method, and 0.1 μg Hg/g for AOAC total Hg method.
[b] NBS Research Material-50; total Hg reference value = 0.95 ± 0.1 μg Hg/g.

total Hg (2). There is no significant difference between the results for the various methods. Essentially all of the Hg present in these fish types (as determined by the AOAC method for total Hg) is in the organic form. This observation has been reported elsewhere for swordfish and tuna (11).

Table 2 presents the results from analysis of selected, fortified products by the AOAC (5, 6) and modified methods with EC detection and by the modified method with MIP detection. There is no significant difference between the results obtained by EC and MIP detection, indicating that there is no significant interference by other electron-capturing compounds at the retention time of methyl mercuric chloride.

Table 2. Comparison of EC and MIP detection in analysis of seafood for methyl mercury by AOAC[a] and modified methods

Commodity	Hg, μg/g: AOAC method/EC[b]	Modified method/EC[c]	Modified method/MIP[d]
Swordfish B	0.76	0.72	0.75
Swordfish C	1.34	1.36	1.36
Tuna B, level 0[e]	0.12	0.16	0.16
Tuna B, level 1[f]	0.92	0.98	0.98
Tuna B, level 2[g]	2.32	2.30	2.25
Tuna C, level 0[e]	NA[h]	0.09	NA
Tuna C, level 1[i]	0.64	0.62	0.65
Oysters, level 0[e]	0.02	0.02[j]	NA
Oysters, level 1[i]	0.52	0.50	0.51
Oysters, level 2[k]	1.42	1.46	1.48

[a] Ref. 3.
[b] Average of 2–4 determinations.
[c] Average of 5 or 6 determinations.
[d] One determination.
[e] Unfortified.
[f] Fortified with 0.803 μg Hg/g.
[g] Fortified with 2.208 μg Hg/g.
[h] NA indicates not analyzed.
[i] Fortified with 0.539 μg Hg/g.
[j] Average of 2 determinations.
[k] Fortified with 1.522 μg Hg/g.

Table 3. Means, standard deviations (SD), and coefficients of variation (CV) for determination of methyl mercury by the modified method

Commodity	Hg, μg/g: Mean[a] ± SD	CV
Swordfish A	1.33 ± 0.05	4
Swordfish B	0.72 ± 0.05	7
Swordfish C	1.36 ± 0.09	7
Shark	2.15 ± 0.05	2
Tuna A	0.55 ± 0.03	5
Tuna B, level 1	0.98 ± 0.02	2
Tuna B, level 2	2.30 ± 0.02	1
Tuna C, level 1	0.62 ± 0.01	2
Oysters, level 1	0.50 ± 0.03	6
Oysters, level 2	1.46 ± 0.04	3

[a] Average of 4–6 determinations.

Coefficients of variation (CVs) for determinations by the modified method with EC detection ranged from 1 to 7% for seafood containing methyl mercury at levels ranging from 0.50 to 2.30 μg Hg/g. These CVs are presented in Table 3 with the corresponding means and standard deviations. Table 4 shows the percent recoveries of methyl mercury from fortified products analyzed by the modified method. The overall average recovery of methyl mercury was 100.5% for the fish types studied. The average recoveries of methyl mercury from shrimp and from oysters at one fortification level were lower than the overall average. We experienced difficulty in homogenizing and mixing the rubbery, gelatinous shrimp homogenate with acetone, toluene, and HCl solution. The oysters fortified at the low level produced emulsions that were not fully eliminated with isopropanol. These difficulties may explain the lower recoveries of methyl mercury from shrimp and oysters.

The cleanup provided by this method is adequate for the determination of methyl mercury at Hg levels >0.25 μg/g.

The quantitation limit (QL) of 0.25 μg Hg/g is based on the height of the methyl mercuric chloride peak (0.005 μg Hg/mL extract) that is equal to 10 times the height of interfering background peaks in chromatograms obtained in this laboratory.

$$QL = (0.005\ \mu g\ Hg/mL\ extract) \times (50\ mL\ extract/g\ sample) = 0.25\ \mu g\ Hg/g$$

Figure 1 shows a typical EC-GC chromatogram for shark extract obtained from analysis by the modified method.

Table 4. Recovery of methyl mercury by the modified method

Commodity	Hg added, μg/g[a]	Av. rec., %[b]
Swordfish A	1.87	109
Shark	1.87	104
Tuna A	1.87	108
Tuna B	0.80	103
Tuna B	2.21	96.8
Tuna C	0.54	99.5
Oysters	0.54	89.6
Oysters	1.52	98.7
Clams	0.93	104
Shrimp	0.93	92.6
Overall av. rec., %		100.5

[a] Fortified with aqueous solution of methyl mercuric chloride.
[b] Average of 2–6 determinations. Recovery for each individual determination was calculated by subtracting average result for unfortified test portions (column 3, Tables 1 and 2) from result for fortified test portion, dividing by μg Hg/g added, and multiplying by 100.

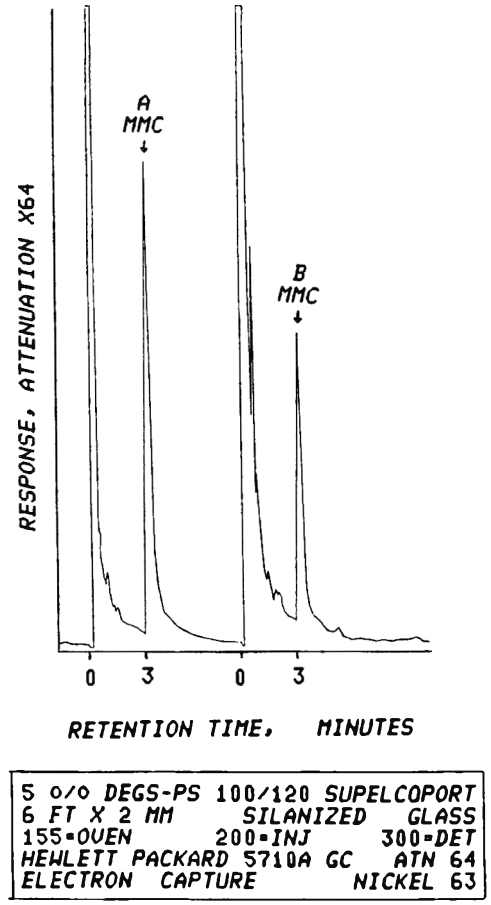

Figure 1. EC-GC chromatograms from determination of methyl mercury in shark tissue: (A) methyl mercuric chloride (MMC) peak from injection of 5 μL standard solution containing 0.0844 ppm Hg; (B) methyl mercuric chloride (MMC) peak from injection of 5 μL extract (2.34 μg Hg/g tissue; 1.09 g tissue/50 mL).

Discussion

Solvent Selection

The first goal of our study was to eliminate the use of benzene for extraction. The U.S. Occupational Safety and Health Administration time-weighted average (TWA) exposure limit for this carcinogen is 10 ppm (12). As a possible substitute for benzene, hexane (isomer mixture, TWA = 100 ppm) was investigated. Hexane (bp 69°C) concentrates rapidly in Kuderna-Danish glassware. Methyl mercuric chloride in hexane gives a short retention time, good peak shape, and steady baselines under the chromatographic conditions in the AOAC official method (5, 6). A major disadvantage of hexane is that it extracts only 23% of the methyl mercuric chloride from the aqueous phase when it is substituted for benzene in the AOAC official method. Hexane, therefore, was eliminated from further consideration.

Toluene (TWA = 100 ppm) was also investigated. The retention time and peak shape of methyl mercuric chloride are almost identical in toluene and benzene under the chromatographic conditions of the AOAC official method. Toluene is also a good solvent because it extracts about 83% of the methyl mercuric chloride in the aqueous phase when it is substituted for benzene in the AOAC method. A disadvantage, however, in using toluene is its high boiling point (111°C) which makes evaporative concentration impossible in Kuderna-Danish glassware on a steam bath. Despite this drawback, toluene was chosen as the solvent to replace benzene, because we felt that the higher quantitation limit which results when evaporative concentration is eliminated (0.25 μg Hg/g) would not prevent using the modified method to enforce the FDA action level for methyl mercury of 1.0 μg Hg/g. Although toluene is safer to use than benzene, it is harmful if inhaled and is flammable. Therefore, all operations should be conducted in a laboratory hood.

Table 5. Determination of methyl mercury in the presence and absence of mercuric chloride by the AOAC and modified methods

Fortification species	Hg added, μg/g	Time of addition	Hg found, μg/g[a] AOAC method (3)	Hg found, μg/g[a] Modified method
None	0		1.50	1.45
Inorganic ($HgCl_2$)	2	after prewash	1.49	1.42
Inorganic ($HgCl_2$)	2	before prewash	1.49	1.51
Organic (methyl mercuric chloride)	4.67,[b] 4.53[c]	before prewash	5.09[d]	4.34[d]

[a] Average of duplicate determinations.
[b] For determination by the AOAC method.
[c] For determination by the modified method.
[d] Net found (fortified concentration found − unfortified concentration found).

Chromatographic Conditions

The reader is referred to previous work done in this laboratory (13) for information on the chromatographic behavior of organomercury compounds on various column-packing materials. The previous study showed that methyl mercuric chloride and ethyl mercuric chloride chromatographed well on diethylene glycol succinate (DEGS) treated with mercuric chloride.

In the work reported here, various column dimensions and percentages of liquid phase were investigated in an effort to shorten elution time. However, no advantage was found for any of the following: 3 ft × 2 mm id column, unsilanized support, and 10% liquid phase load. Therefore, we decided to continue using the chromatographic conditions in the AOAC official method (5, 6): 6 ft × 2 mm id silanized glass column packed with 5% DEGS-PS on 100–120 mesh Supelcoport. Our EC chromatographic system gave a linear response for 5 μL injections of standard solutions containing 0.005–0.300 μg Hg/mL (correlation coefficient = 0.99991).

Optimization of Extraction Parameters

Preliminary experiments showed that the amount of methyl mercury extracted from fish tissue by toluene increases when the volume of HCl solution is decreased and the volume of toluene is increased. This effect is explained by the fact that methyl mercuric chloride is slightly soluble in water (14).

Consequently, a series of experiments was conducted to optimize conditions for maximum recovery of methyl mercury. Results showed that maximum recovery is obtained when methyl mercury in 1 g fish tissue (prewashed with acetone and toluene) is extracted with 2.5 mL HCl solution and 20 mL toluene. Under these conditions, 95% of the methyl mercury in the aqueous phase is transferred to the organic phase by one extraction.

Swordfish was chosen for the optimization experiments because FDA monitoring programs have shown that it frequently contains high levels of methyl mercury. A 1 g test portion was chosen because a smaller quantity might have caused homogeneity problems and would have resulted in a

higher quantitation limit. A larger weight would have required using more organic solvent, which we wanted to keep at a minimum. All volumes of HCl solution were ≥2.5 mL, because initial experiments showed that smaller quantities did not provide sufficient mixing with the 1 g test portion. A 20 mL portion of toluene was used because initial experiments showed that larger volumes could not be adequately mixed in the equipment that was readily available in our laboratory.

Shaking time.—The effect of shaking the acidified sample with toluene for various times was studied in an effort to reduce the time required to complete the analysis. Shaking times investigated were 2, 5, 10, and 15 min. A 1 g portion of swordfish was prewashed with acetone and toluene, and the methyl mercury was extracted once with 2.5 mL HCl solution and 20 mL toluene. All mixtures were mechanically shaken.

Experimental results showed that the length of shaking time has no significant effect on the recovery of methyl mercury from swordfish. However, past experience in our laboratory indicated that mixtures from some products such as homogenized shrimp do not appear to be well mixed after 2 min on a mechanical shaker. On the basis of this experience, we chose the 5 min shaking time when a mechanical shaker is used. Mixtures shaken thoroughly by hand appeared to be well mixed after 2 min, and the analytical results were equivalent to those obtained after 5 min of mechanical shaking.

Number of extractions.—When the optimized analytical parameters are used, only 2 solvent extractions are necessary for acceptable recovery. The extraction optimization experiments show that about 95% of the methyl mercury is transferred to toluene in the first extraction when the optimized analytical parameters are used. It follows that 99.75% [95% + (95% of 5%) = 99.75%] of the methyl mercury will be transferred to toluene in 2 combined extractions. This recovery is well within the experimental precision of the method. For analysis of 2 combined 20 mL extracts diluted to 50.0 mL, the quantitation limit of the method is 0.25 μg Hg/g. Although this limit is higher than the 0.05 μg Hg/g quantitation limit of the official method (5, 6), it is adequate for monitoring at the 1.0 ppm action level.

Methyl Mercury in the Presence of Inorganic Mercury

Several experiments were carried out to determine whether the modified method is specific for methyl mercury when inorganic Hg (mercuric chloride) is present. Swordfish containing about 1.5 μg Hg/g was used for the experiment. The fish was fortified with solutions of mercuric chloride and methyl mercuric chloride in water. Some portions were fortified with mercuric chloride after the solvent prewashes to determine whether mercuric chloride is extracted into the toluene and interferes with the methyl mercuric chloride peak in the chromatogram. Other portions were fortified with mercuric chloride before the solvent prewashes to determine whether mercuric chloride can be removed by the prewash if it does interfere with methyl mercury. A third set was fortified with methyl mercuric chloride before prewashing to determine whether it is lost during the prewash step. The experimental results are presented in Table 5. There is no significant difference between the results for the unfortified portions and those for the portions fortified with inorganic Hg. The results show that inorganic Hg does not interfere with the analysis for methyl mercury and that methyl mercury is not lost during the prewash steps.

During routine analysis, the analyst may fortify test portions with methyl mercuric chloride between the prewash steps and the addition of HCl solution as directed in the procedure. We have obtained excellent recoveries (95–106%) using this time-saving step.

MIP Detection of Methyl Mercury

Because the EC detector employed in the modified method responds to many electron-capturing compounds, we decided to monitor the GC effluent with an Hg-specific detector. We compared the Hg concentrations in the extracts analyzed by obtaining responses with the EC and MIP detectors. If the EC result had been greater than the MIP result for the same extract, coeluting compounds (compounds other than methyl mercuric chloride) would have been indicated. Since the EC and MIP results were equal, there was no coeluting interference.

The MIP output was not linear with respect to the Hg concentration of standard solutions at the higher levels. MIP results from the analysis of test samples, therefore, were calculated by using a separate set of standard solutions with concentrations of Hg that more closely matched the Hg concentrations in the test samples. (For the analyses with MIP detection, methyl mercuric chloride peak heights in chromatograms of standard solutions were within 5–8% of the peak heights in chromatograms of extracts.) Results were calculated by comparing the average peak height from duplicate injections of test extract to the average peak height from 2 injections of standard solution made immediately before and 2 injections of standard solution made immediately after the injections of test extract. This 6 injection sequence was necessary to monitor the MIP system stability, which was frequently disrupted by laboratory environmental changes (temperature, pressure, vibration, etc.). If the range of the 4 standard peak heights was >10% of the average standard peak height, the data were rejected and the entire 6 injection sequence was repeated.

The MIP system response (at Hg emission line 253.45 nm) for potentially interfering amounts of Cl, C, and P was evaluated by comparing the MIP response to hexachlorobenzene (C_6Cl_6, 2.05 retention time relative to methyl mercuric chloride) and phorate ($C_7H_{17}O_2PS_3$, 3.48 relative retention time) with the MIP response to methyl mercuric chloride. Cl, C, and P do not produce an MIP response at respective levels that are 1340, 1510, and 556 times as great as the Hg level in the sample extracts analyzed. Higher levels of Cl, C, and P were not investigated.

MIP-GC and EC-GC analysis of selected extracts gave equivalent results, as shown in Table 2. Therefore, we conclude that the modified method provides adequate cleanup for the determination of methyl mercury in seafood.

Conclusions

The method developed in this study gives accurate and precise results for the determination of methyl mercury in fish and shellfish. Coefficients of variation obtained by this method range from 1 to 7% for seafood containing 0.50–2.30 μg Hg/g. The overall average recovery is 100.5% (range 89.6–109%) for test samples fortified to contain 0.56–4.05 μg Hg/g (values from Table 4 plus appropriate values from column 3, Table 1 or 2). This modified method has several advantages over the AOAC official method in that it (*1*) eliminates the health hazard associated with benzene by replacing it with toluene; (*2*) reduces analytical time by 30–45 min per determination by eliminating evaporative concentration and reducing the number of extractions, quantitative transfers, and extract injections; and (*3*) is easier to use with less chance of low recoveries by reducing the number of quantitative

transfers. The parameters for extracting methyl mercury into toluene are optimized so that 99% of the methyl mercury is recovered. Experiments show that mercuric chloride does not interfere and that methyl mercury is not lost during analysis.

We are currently conducting an interlaboratory study to evaluate and possibly recommend the modified method for AOAC official first action status.

Acknowledgment

The authors thank Lee Miller, FDA, Division of Food Chemistry and Technology, for setting up and maintaining the MIP detection system.

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MICROBIOLOGICAL METHODS

Glucuronidase Assay in a Rapid MPN Determination for Recovery of *Escherichia coli* from Selected Foods

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Glucuronidase is present in most strains of *Escherichia coli* but absent in most other enteric microorganisms; therefore, an assay for this enzyme is useful for determining the presence of the organism. The substrate 4-methylumbelliferyl beta-D-glucuronide (MUG) is incorporated into either lauryl tryptose (LT) broth or EC medium; the inoculated tubes are then incubated under specified conditions and examined under longwave UV light for the presence of a fluorogenic glucuronidase end product. When compared with the 10-day most probable number (MPN) procedure of AOAC, the LT-MUG and the EC-MUG tests required 24 and 96 h, respectively, and gave comparable mean log MPN values for samples of crabmeat, sunflower kernels, and walnut pieces. However, false-positive and false-negative reactions were observed with foods tested by both of these rapid methods. Overall, method sensitivity was not compromised by using the LT-MUG rather than the EC-MUG method. Incorporation of 25 μg MUG/mL into LT broth resulted in diminished fluorescence of positive reactions, whereas MUG concentrations of 50 and 100 μg/mL provided decisive fluorogenic reactions.

The presence of *Escherichia coli* in foods is of regulatory significance because it indicates fecal contamination and, with some strains of this bacterium, the presence of a pathogen. The widely used most probable number (MPN) method of AOAC enumerates coliforms on the basis of an organism's ability to ferment lactose (1). Serial 10-fold dilutions of the food sample are inoculated into tubes of lauryl tryptose (LT) broth. After incubation at 35°C, positive (presence of gas) tubes are subcultured to EC medium and incubated at 45.5°C. Tubes of EC medium with gas are considered to contain fecal coliforms. The AOAC method requires up to 4 days for determination of fecal coliforms and an additional 6 days if confirmation of these organisms as *E. coli* is desired.

The fluorogenic assay for the enzyme glucuronidase substantially reduces the analytical time needed to obtain an *E. coli* MPN. In one study this enzyme was observed in 97% of the *E. coli* strains tested but not in strains of most other enteric genera (2). To test for glucuronidase, its substrate 4-methylumbelliferyl beta-D-glucuronide (MUG) is incorporated into the appropriate medium, and after incubation of the inoculated tubes, the presence of a fluorogenic glucuronidase end product is observed under longwave UV light. The objective of this study was to determine if recoveries of *E. coli* obtained by a rapid MPN method incorporating MUG into either LT broth or EC medium would equal those obtained by the lengthier AOAC method.

Experimental

Inoculation of Samples

Brain heart infusion (BHI) broth cultures of *E. coli* ATCC 9637, 11775, and 25922 that were incubated from 18 to 24 h at 35°C were washed twice with Butterfield's phosphate buffer and serially diluted. Appropriate dilutions to be used as inocula for sunflower kernels and walnut pieces were heat-shocked at 55°C for 10 min. Cultures were further diluted to a final volume of 1 L and shaken manually. Portions (1000 g) of these 2 foods were submerged for 2 min in 1 L diluted shocked cell suspension in a sterile stomacher bag. The foods were drained and then dried for 18–24 h at 35°C. These 2 types of inoculated foods were aged 1–3 months before being analyzed.

Inocula for pasteurized backfin crabmeat were not heat-shocked, because any *E. coli* organisms naturally occurring in this product most likely would be introduced after pasteurization and would not be subjected to thermal stress. Accordingly, BHI broth cultures grown as described above were washed twice and used to inoculate crabmeat just before analysis.

Microbiological Analysis

The protocol used to determine the efficiency of incorporating MUG into LT broth or EC medium is shown in Figure 1. For each experiment, 3 replicates, each weighing 50 g, were withdrawn from the 1000 g amounts of inoculated sunflower kernels or walnuts. For the crabmeat, three 50 g replicates were inoculated with the unstressed cells as described above. Replicates were blended in a Waring commercial blender for 2 min at 14 000 rpm. Serial 10-fold dilutions were made and inoculated into LT and LT-MUG broth. For purposes of discussion, 4 methods are delineated. For the first method, recommended by AOAC, all LT tubes showing gas at 35°C after 24 and 48 h incubation were subcultured to EC medium. Inoculated EC tubes were incubated in a waterbath at 45.5°C; at 24 and 48 h incubation intervals, tubes with gas were streaked to EMB agar. Colonies typical of *E. coli* were confirmed by indole, methyl red, Voges-Proskauer, and citrate utilization (IMViC) reactions, Gram stain, and ability to produce gas when the pure culture was reinoculated into LT broth.

For the second and third methods, designated LT (24 h) to EC-MUG and LT (48 h) to EC-MUG, all tubes of LT broth, regardless of fermentative reaction, were subcultured to EC-MUG broth at 24 and 48 h, respectively. Because the MUG test is based on a fluorogenic rather than a fermentative reaction, all tubes of LT broth were subcultured to EC-MUG broth. Tubes of inoculated EC-MUG media were incubated in a waterbath at 45.5°C, and at 24 and 48 h intervals, tubes were examined for fluorescence under longwave UV light at a wavelength of 366 nm. All tubes were subsequently streaked to Levine EMB agar. Suspect *E. coli* colonies were confirmed as described for the AOAC method.

For the fourth method, serial dilutions of the food were inoculated into LT-MUG broth, incubated for 24 h at 35°C, and examined for fluorescence as described previously. All LT-MUG tubes were subcultured to EC medium, incubated in a waterbath at 45.5°C, and streaked to EMB agar at 24 and 48 h intervals. Suspect *E. coli* colonies were confirmed as described for the AOAC method.

If present, MUG was used at a concentration of 100 μg/mL medium. Subsequently, however, a comparison was made of 3 concentrations of MUG (25, 50, and 100 μg/mL) incorporated into LT broth. Whether used in LT broth or in

Received March 10, 1986. Accepted April 19, 1986.

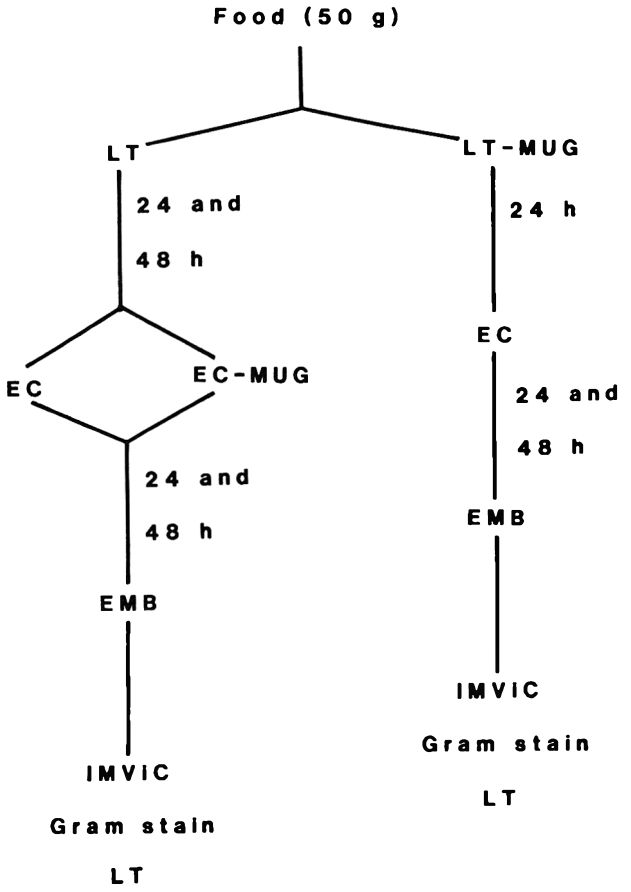

Figure 1. Protocol for comparing AOAC and fluorogenic MPN methods for *E. coli*.

EC medium, appropriate amounts of MUG were dissolved in warm water and added to either of these 2 media. In preparing the LT-MUG broth or EC-MUG medium, compensation was made for the amount of water used to dissolve the MUG. After preparation, the LT and LT-MUG broths and the EC and EC-MUG media were dispensed into tubes containing inverted fermentation vials and sterilized by autoclaving.

Statistical Analysis

Each MPN value was converted to the log base 10, and the mean log value for the replicates analyzed by each method was calculated. For each food, an analysis of variance was made of the log-transformed data, and Duncan's multiple comparison procedure (3) was used to assess pairwise differences between the method mean log values at $\alpha = 0.05$.

Results and Discussion

The incorporation of MUG into LT broth for the rapid detection of *E. coli* in foods has been reported elsewhere (4–7). Koburger and Miller (8), however, reported that the use of LT-MUG broth for analyzing oysters resulted in a large number of false-positive reactions because of the endogenous presence of glucuronidase in this food. Incorporation of MUG into EC medium, rather than into LT broth, reduced this interference and resulted in an efficient, rapid test for *E. coli*. Accordingly, LT-MUG and EC-MUG media were tested for their ability to determine the presence of *E. coli* in specific foods (Table 1) for which the Food and Drug Administration (FDA) has regulatory responsibility.

With the LT-MUG method, results are available 24 h after initiation of analysis. When MUG is incorporated into EC medium, however, subcultures are made from the LT broth to the EC-MUG medium at 24 and 48 h. These methods are known as the LT (24 h) to EC-MUG and LT (48 h) to EC-MUG methods, respectively (Table 1).

Table 1. Relative efficiency of incorporating MUG into LT broth and EC medium for the enumeration of *E. coli* in selected foods

Trial	Mean $\log_{10}$ *E. coli* MPN values/g[a]			
	AOAC	LT-MUG	LT (24 h) to EC-MUG	LT (48 h) to EC-MUG
	Crabmeat			
1	−0.265	1.500	2.707[b]	2.186[b]
2	1.745	1.529	1.745	1.655
	Sunflower kernels			
1	1.786	1.745	1.786	1.786
2	2.412	2.231	2.412	2.412
	Walnut pieces			
1	−0.523	−0.470	−0.496	−0.496
2	0.983[c]	−0.341	0.227[c]	0.227[c]

[a] MPN values were determined for 3 replicate samples for each method.
[b] Significantly greater than AOAC mean log value.
[c] Significantly greater than LT-MUG mean log value.

For the first trial with crabmeat, the mean log MPN values obtained with both EC-MUG methods were significantly higher than those obtained with the AOAC method. However, the mean log value obtained with the LT-MUG method did not differ significantly from that obtained with the AOAC method or with either EC-MUG method. For the second trial with crabmeat, there were no significant differences among the mean log MPN values obtained by all 4 methods.

Of the 3 types of foods used in this study, the results with sunflower kernels showed the closest agreement of mean log MPN values obtained with the 4 methods. For both trials, there were no significant differences among the mean log values obtained by any of the methods. Moreover, the mean log values obtained by the AOAC and EC-MUG methods were identical in both trials.

For the first trial with walnut pieces, there were no significant differences among the mean log values obtained with the 4 methods. For the second trial, the mean log values of the AOAC method and both EC-MUG methods were significantly higher than that given by the LT-MUG method.

A comparison of the mean log MPN values of the LT-MUG and AOAC methods for all 6 trials collectively showed only one instance (second trial with walnuts) of a significant difference in means. Similarly, in a comparison of the EC-MUG and AOAC methods for all 6 trials, there was a significant difference in means in only one instance (first trial with crabmeat). When the LT-MUG was compared with the EC-MUG methods, there was only one instance (second trial with walnut pieces) in which the mean log values were significantly different. These results indicate that the LT-MUG and EC-MUG methods gave comparable *E. coli* MPN values for the foods examined in this study. Moreover, the mean log MPN values obtained by the 2 EC-MUG methods were identical in 4 of 6 trials and showed no significant difference in the other 2 trials. Thus, for the EC-MUG methods, there was no apparent advantage for making a 48 h subculture in addition to the 24 h subculture from the LT broth to the EC-MUG medium.

Table 2 shows the number of discrepant reactions obtained with the fluorogenic methods, i.e., the numbers of false-positive and false-negative reactions with respect to fluorescence

Table 2. Correlation of fluorescence and the presence of *E. coli* in selected foods when MUG is incorporated into LT broth and EC medium

Method	Reactions of media containing MUG			No. of tubes *E. coli*	
	Growth	Gas	Fluorescence	Recd (+)	Not recd (−)
Crabmeat					
LT-MUG	+	+	+	38	10[a]
	+	+	−	0	0
	+	−	+	0	0
	+	−	−	0	11
	−	−	+	0	0
	−	−	−	0	49
LT (24 h) to EC-MUG	+	+	+	36	0
	+	+	−	0	0
	+	−	+	8	0
	+	−	−	0	0
	−	−	+	3	1[a]
	−	−	−	2[b]	58
LT (48 h) to EC-MUG	+	+	+	35	0
	+	+	−	0	0
	+	−	+	2	0
	+	−	−	0	0
	−	−	+	3	0
	−	−	−	8[b]	60
Sunflower kernels					
LT-MUG	+	+	+	47	0
	+	+	−	1[b]	2
	+	−	+	0	0
	+	−	−	0	46
	−	−	+	0	0
	−	−	−	0	45
LT (24 h) to EC-MUG	+	+	+	50	0
	+	+	−	0	0
	+	−	+	0	0
	+	−	−	0	0
	−	−	+	0	0
	−	−	−	0	94
LT (48 h) to EC-MUG	+	+	+	50	0
	+	+	−	0	0
	+	−	+	0	0
	+	−	−	0	0
	−	−	+	0	0
	−	−	−	0	94
Walnut pieces					
LT-MUG	+	+	+	2	4[a]
	+	+	−	0	0
	+	−	+	4	26[a]
	+	−	−	0	43
	−	−	+	0	0
	−	−	−	0	65
LT (24 h) to EC-MUG	+	+	+	9	0
	+	+	−	0	0
	+	−	+	1	0
	+	−	−	0	17
	−	−	+	0	0
	−	−	−	0	118
LT (48 h) to EC-MUG	+	+	+	8	0
	+	+	−	0	0
	+	−	+	0	0
	+	−	−	1[b]	12
	−	−	+	0	0
	−	−	−	0	123

[a] False-positive reactions (*E. coli* not recovered from fluorescent-positive tubes).
[b] False-negative reactions (*E. coli* recovered from fluorescent-negative tubes).

of the MUG-containing medium. A false-positive reaction is one in which the LT-MUG or EC-MUG medium fluoresced, but *E. coli* was not recovered. A false-negative reaction is one in which the LT-MUG or EC-MUG medium did not fluoresce, but *E. coli* was recovered.

Table 3. Relative efficiency of 3 concentrations of MUG incorporated into LT broth for the enumeration of *E. coli* in crabmeat

Method	MUG, μg/mL	Mean $\log_{10}$ *E. coli* MPN values/g[a]	
		Trial 1	Trial 2
AOAC	—	1.196	3.038
LT-MUG	25	1.259	3.327
LT-MUG	50	1.412	3.255
LT-MUG	100	1.106	3.219

[a] MPN levels were determined for 3 replicates for each method.

For crabmeat, 10 LT-MUG tubes gave a +, +, + reaction (i.e., growth (+), gas production (+), and fluorescence (+), respectively), but *E. coli* was not recovered (false-positive reactions). The LT (24 h) to EC-MUG method gave one false-positive (−, −, +) and 2 false-negative (−, −, −) reactions. The most serious discrepancy was the occurrence of 8 false-negative reactions with the LT (48 h) to EC-MUG method (−, −, −). These tubes showed no growth, gas, or fluorescence, but *E. coli* was recovered from them.

Sunflower kernels had the least number of discrepant reactions. Only one false-negative reaction was obtained with the LT-MUG method (+, +, −).

For walnut pieces, the LT-MUG gave false-positive reactions with the +, +, + combination (4 tubes) and the +, −, + combination (26 tubes). The only other discrepant reaction was a single false-negative reaction given by the LT (48 h) to EC-MUG method (+, −, − combination).

Based on the results in Tables 1 and 2, we decided to investigate further the LT-MUG method rather than the EC-MUG methods. Table 1 showed that the LT-MUG and EC-MUG methods were comparable, with the exceptions noted earlier, for the enumeration of *E. coli* in the foods used for this investigation. Moreover, there were no major differences in the occurrence of discrepant reactions among the methods

Table 4. Correlation of fluorescence and the presence of *E. coli* in crabmeat when various concentrations of MUG are incorporated into LT broth

MUG, μg/mL	Reactions of media containing MUG			No. of tubes *E. coli*	
	Growth	Gas	Fluorescence	Recd (+)	Not recd (−)
25	+	+	+	46	6[a]
	−	+	−	5[b]	1
	−	−	+	0	0
	−	−	−	0	7
	−	−	+	0	0
	−	−	−	0	43
50	+	+	+	50	6[a]
	+	+	−	0	0
	+	−	+	0	0
	+	−	−	0	6
	−	−	+	0	0
	−	−	−	2[b]	44
100	+	+	+	49	7[a]
	+	+	−	0	0
	+	−	+	0	0
	−	−	−	0	9
	−	−	+	0	0
	−	−	−	0	43

[a] False-positive reactions (*E. coli* not recovered from fluorescent-positive tubes).
[b] False-negative reactions (*E. coli* recovered from fluorescent-negative tubes).

except for the relatively high number of false-negative reactions by the LT (48 h) to EC-MUG method for crabmeat and the relatively high number of false-positive reactions by the LT-MUG method for crabmeat and walnut pieces. From the standpoint of assuring microbiological quality, the occurrence of false-negative reactions would be a more serious shortcoming than the occurrence of false-positive reactions.

Eventual adoption of the LT-MUG rather than an EC-MUG method offers the major advantage of reducing analytical time. Because there is no need to initially inoculate and incubate the dilutions in LT broth before subculturing to EC-MUG medium, up to 2 days are saved. Another advantage is that the use of an elevated temperature is avoided. The use of surfactants and elevated incubation temperatures has resulted in a loss of plasmids responsible for virulence factors in *E. coli* (9). Thus, enrichment under such conditions could reduce the ability to recover pathogenic strains of *E. coli*. Although LT broth does contain the surfactant sodium lauryl sufate, incubation at 45.5°C is avoided. The relative significance of these 2 factors (i.e., presence of surfactants and incubation at elevated temperatures) on plasmid-mediated toxigenicity of *E. coli* remains to be determined.

Because of the high cost of the MUG compound, we attempted to incorporate MUG into LT broth at concentrations lower than 100 μg/mL (Table 3). For 2 separate trials with crabmeat, no significant differences were observed in the mean log MPN values obtained with the AOAC method and the LT-MUG method using 3 concentrations of MUG.

Table 4 shows the false-positive and false-negative reactions obtained when MUG was incorporated at 3 different concentrations into LT broth. At 25, 50, and 100 μg MUG/mL, there were 6, 6, and 7 false-positive (+, +, +) reactions, respectively: 25 μg MUG/mL gave 5 false-negative (+, +, −) reactions; 50 μg MUG/mL gave 2 false-negative (−, −, −) reactions. No false-negative reactions were observed for 100 μg MUG/mL LT broth. Despite these false-negative reactions, there was no significant difference in the mean log MPN values for 25, 50, and 100 μg MUG/mL LT broth. However, the intensity of fluorescence for a positive reaction diminished significantly when 25 μg MUG/mL was used. Moreover, reading of the LT-MUG broth tubes was subjective at this concentration. When MUG was used at 50 or 100 μg/mL, the fluorescent reactions were decisive.

One problem with the MUG test is that autofluorescence may arise from either or both of 2 sources. The first source is the glass test tube that contains the MUG broth media. All glass tubes must be screened for autofluorescence before dispensing the broth media to minimize false-positive reactions. The problem of a particular lot of test tubes exhibiting autofluorescence may be eliminated by boiling the tubes in a 5% sodium nitrate solution. The second source of autofluorescence is the endogenous presence of glucuronidase in certain foods. The extent of this problem other than in oysters (8) is not yet known.

Preliminary results from this study agree with other evaluations of the MUG test (4–7); however, further investigation of the usefulness of this method seems warranted. It would be premature at this point to recommend the LT-MUG method as an alternative to the conventional MPN method of the AOAC. We are currently testing the efficiency of the LT-MUG method for analysis of foods naturally contaminated with *E. coli*.

Acknowledgments

The authors thank Stephen W. Butler and Foster D. McClure, Division of Mathematics, FDA, for statistical analysis of the data and Lois Tomlinson for preparation of the final version of this manuscript.

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Most Probable Number Method for Isolation and Enumeration of *Staphylococcus aureus* in Foods: Collaborative Study

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Enumeration of *Staphylococcus aureus* in foods was coilaboratively studied by comparing the present AOAC final action method, 46.062, which uses trypticase soy broth with 10% NaCl to a proposed replacment method which uses the same broth with 1% sodium pyruvate added. Fifteen collaborators analyzed uninoculated samples of milk, tuna salad, and ground turkey, as well as samples inoculated with low (10^2 cells/g), middle (10^4 cells/g), and high (10^6 cells/g) levels of *S. aureus*. The samples were frozen immediately to maintain the inoculated level of *S. aureus* in the food. A different strain of *S. aureus* was used for each food; heat-stressed *S. aureus* cells were used to inoculate the milk samples. The pyruvate-amended broth significantly ($\alpha = 0.05$) increased enumeration of low, middle, and high levels of *S. aureus* from milk and ground turkey, and from tuna salad at middle and high levels. The pyruvate-amended media method has been adopted official first action to replace method 46.062.

Staphylococcus aureus has been well documented as a cause of foodborne disease (1, 2). In addition, the organism is often used as an indicator of sanitation because it is easily destroyed by various sanitizers and heat treatments (3).

Two methods have been collaboratively studied and adopted by AOAC as official final action methods (4). One is a most probable number (MPN) technique using trypticase soy broth with 10% NaCl (TSBS) as the primary recovery medium. However, TSBS has been shown to be inhibitory to the recovery of stressed cells of *S. aureus* (5). Even though TSBS is inhibitory, its use is recommended for analyzing raw food ingredients and nonprocessed foods expected to contain a large population of competing species because the medium is highly selective for *S. aureus* (4). In addition, the medium is recommended for use in routine surveillance of products for sanitary quality in which <100 *S. aureus* cells/g may be expected (3).

Previous efforts to develop a different recovery medium with selective abilities equal to TSBS have not been successful (6, 7). Research to reduce the damaging effect of 10% NaCl on stressed *S. aureus* cells led to the addition of 1% sodium pyruvate to TSBS (PTSBS). Pyruvate acts to prevent cell death from hydrogen peroxide accumulation during aerobic growth and repair (8). The Associate Referee has compared TSBS and PTSBS, and found that pyruvate increased recovery of both heat-stressed and unstressed *S. aureus* cells (9). Therefore, a comparative collaborative study was undertaken to compare recovery of both stressed and nonstressed *S. aureus* in TSBS (Method A) with recovery by the same medium with 1% sodium pyruvate added (Method B).

Submitted for publication March 17, 1986.
This report of the Associate Referee, G. A. Lancette, was presented at the 99th AOAC Annual International Meeting, Oct. 27–31, 1985, Washington, DC.
The recommendation of the Associate Referee was approved by the General Referee and the Committee on Microbiology and was adopted by the Association. See the General Referee and Committee reports, *J. Assoc. Off. Anal. Chem.* (1987) **70**, March issue.

Collaborative Study

Microbiologists in 15 laboratories participated as collaborators. Each received an outline of the collaborative study, a copy of the methods, and data reporting forms. Materials used in the study were also supplied by the Associate Referee to avoid possible variations in results from the use of different materials. Each collaborator analyzed duplicate samples of uninoculated milk, tuna salad, and ground turkey, and samples inoculated with low, middle, and high levels of *S. aureus*. Each food was inoculated with a different strain of *S. aureus* taken from the Center for Microbiological Investigations (CMI) culture file (Table 1). One additional set of samples was analyzed by CMI as the control laboratory.

The test samples were prepared by adding various amounts of pH 7.2 buffered phosphate diluent seeded with an *S. aureus* culture. A 24 h culture in trypticase soy broth incubated at 35°C was centrifuged at 30 877 × *g* for 10 min. Cells were resuspended in pH 7.2 phosphate buffer.

Milk samples were inoculated with heat-stressed cells of strain 547. An initial 1:10 dilution of cells in phosphate diluent was heated at 56°C in a gyrating water bath (100 cycles/min) for 15 min and cooled in an ice bath for 10 min. Cells were further diluted 1:100 and 1:10 000, and were used to seed the samples to obtain low ($\leq 10^2$ cells/mL), middle ($\leq 10^4$ cells/mL), and high ($\leq 10^6$ cells/mL) levels of *S. aureus* in milk on the day of sample analysis.

Tuna salad and ground turkey were inoculated with unheated cell suspensions diluted 1:100, 1:1000, and 1:100 000, which were used to seed samples at low ($\leq 10^2$ cells/g), middle ($\leq 10^4$ cells/g), and high ($\leq 10^6$ cells/g) levels of *S. aureus*.

Duplicate portions of each batch of contaminated product and the uninoculated batch were transferred aseptically to Whirlpak bags and frozen at −20°C. Samples were shipped frozen to each laboratory in a Styrofoam-insulated container. Samples were held frozen until analysis began. Collaborators were asked to begin analysis of milk the first week and tuna salad and ground turkey samples the second week of the study.

METHODS

Method A

AOAC **46.062** (4).

Table 1. *S. aureus* cultures used for collaborative study

Strain[a]	Source	Food inoculated	Enterotoxins produced
547	cheddar cheese	milk	A, D
493	tuna salad	tuna salad	A, B
F42	stool	ground turkey	A, C, E

[a] *S. aureus* strains obtained from Center for Microbiological Investigations culture file.

Table 2. Collaborative results on comparison of media used in method for counting (log_{10}) *S. aureus* strain 547 in milk

	Low level				Mid level				High level				Uninoculated			
	TSBS		PTSBS		TSBS		PTSBS		TSBS		PTSBS		TSBS		PTSBS	
Coll.	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
1	2.97	1.97	3.36	2.97	3.64	3.97	4.63	4.36	4.63	4.97	5.66	5.66	<0.48	<0.48	<0.48	0.48
2	0.96	1.63	2.97	2.63	3.36	3.87	4.36	4.97	5.38	5.38	5.66	5.38	<0.48	<0.48	<0.48	0.48
3	3.17	2.63	2.97	2.63	4.36	4.32	4.63	5.38	5.18	5.38	6.04	5.66	<1.48	<1.48	<1.48	<1.48
4	2.36	1.97	2.36	1.97	3.97	3.36	4.08	3.36	4.97	4.36	5.38	4.36	<0.48	<0.48	<0.48	<0.48
5	1.86	1.96	2.63	2.63	3.64	3.97	4.36	4.38	4.61	4.32	5.38	5.18	<0.48	<0.48	<0.48	<0.48
6	1.63	1.97	3.36	2.63	3.36	2.97	4.63	4.36	3.97	3.97	6.04	5.66	<0.48	<0.48	<0.48	<0.48
7	1.88	1.63	1.97	1.97	4.36	3.08	3.64	3.97	3.97	4.59	3.97	4.63	<0.48	<0.48	<0.48	<0.48
8	1.36	1.88	2.63	2.97	3.36	3.36	5.38	4.36	4.63	4.36	5.38	5.38	<0.48	<0.48	<0.48	<0.48
9	1.48	1.96	1.96	2.63	2.63	1.55	3.97	4.18	3.36	2.97	5.66	4.97	<1.48	<1.48	<1.48	<1.48
10	2.36	2.63	2.97	3.36	3.97	4.63	4.63	4.63	5.38	5.66	5.66	5.66	<0.48	<0.48	<0.48	<0.48
11	1.88	1.97	2.63	2.97	3.36	3.36	4.18	4.18	4.36	5.66	5.66	5.66	<0.48	0.56	<0.48	1.97
12	2.36	2.08	2.97	2.97	4.36	4.63	5.38	4.63	5.38	6.04	6.04	6.04	<0.48	<0.48	<0.48	<0.48
13	1.55	1.55	2.36	2.36	2.36	2.97	3.97	4.63	4.59	4.63	4.81	5.38	<1.48	<1.48	<1.48	<1.48
14	2.36	2.63	2.36	3.36	3.36	3.64	4.63	4.97	5.38	4.97	5.66	6.04	<0.48	<0.48	<0.48	<0.48
15	1.97	1.63	2.36	2.63	2.97	3.36	4.97	3.97	3.36	4.36	4.36	5.38	<0.48	<0.48	<0.48	<0.48
16	2.18	1.97	2.88	2.36	3.64	3.97	4.36	4.97	5.18	4.97	6.04	6.04	<0.48	<0.48	<0.48	<0.48
n_L[a]	16		16		16		16		16		16					
$\bar{x}$	2.01		2.68		3.55		4.47		4.71		5.45					
S_L	0.3637		0.2647		0.5425		0.2590		0.6185		0.4150					
CV_L, %	7.86		4.29		6.63		2.51		5.69		3.30					
S_o	0.3048		0.3182		0.3968		0.4057		0.3816		0.3485					
CV_o, %	6.58		3.47		4.85		3.94		3.51		2.78					
S_r	0.1057		0.0869		0.1527		0.0846		0.1687		0.1206					
S_x	0.4746		0.4139		0.6721		0.4791		0.7267		0.5419					
CV_x, %	10.25		6.71		8.22		4.65		6.69		4.31					

[a] Statistical evaluation: n_L = number of laboratories used in computations; $\bar{x}$ = overall average; S_L = between-laboratory standard deviation; CV_L = between-laboratory coefficient of variation; S_o = repeatability standard deviation; CV_o = repeatability coefficient of variation; S_x = standard deviation for overall average; S_x = reproducibility standard deviation; CV_x = reproducibility coefficient of variation.

Method B

Staphylococcus aureus in Foods
Most Probable Number Method for
Isolation and Enumeration
First Action

(Applicable to detection and enumeration of small numbers of *S. aureus* in food ingredients and food expected to contain large population of competing species)

Apparatus

(a) *Pipets.*—1.0 mL with 0.1 mL graduations; 5.0 mL and 10.0 mL with 0.5 and 1.0 mL graduations.

(b) *Blender.*—Waring Blendor, or equiv., 2-speed model, with high-speed operation at 16 000–18 000 rpm, and 1 L glass or metal blender jars with covers. One jar is required for each analytical unit.

(c) *Mixer.*—Vortex Genie, or equiv.

(d) *Water bath.*—Maintained at 35–37°.

(e) *Incubator.*—Maintained 35°.

Media and Reagents

(a) *Trypticase (tryptic) soy broth with 10% sodium chloride and 1% sodium pyruvate.*—Add 95 g NaCl to 1 L soln of 17.0 g trypticase or tryptose (pancreatic digest of casein), 3.0 g phytone (papaic digest of soya meal), 5.0 g NaCl, 2.5 g K_2HPO_4, 2.5 g dextrose (dehydrated trypticase or tryptic soy broth is satisfactory), and 10 g sodium pyruvate. Adjust to pH 7.3. Heat gently if necessary. Dispense 10 mL into 16 × 150 mm tubes. Autoclave 15 min at 121°. Final pH, 7.3 ± 0.2. Store ≤1 month at 4 ± 1°.

(b) *Physiological salt soln.*—Dissolve 8.5 g NaCl in 1 L H_2O. Autoclave 15 min at 121° and cool to room temp.

(c) *Baird-Parker medium (egg tellurite glycine pyruvate agar, ETGPA).*—*(1) Basal medium.*—Suspend 10.0 g tryptone, 5.0 g beef ext, 1.0 g yeast ext, 10 g Na pyruvate, 12.0 g glycine, 5.0 g $LiCl \cdot 6H_2O$, and 20.0 g agar in 950 mL H_2O. Heat to bp with frequent agitation to dissolve ingredients completely. Dispense 95 mL portions into screw-cap bottles. Autoclave 15 min at 121°. Final pH, 7.0 ± 0.2 at 25°. Store ≤1 month at 4 ± 1°.

(2) Enrichment.—Bacto EY tellurite enrichment (Difco Laboratories) or prep. as follows: Soak fresh eggs ca 1 min in diln of satd $HgCl_2$ soln (1 + 1000). Aseptically crack eggs and sep. yolks from whites. Blend yolk and physiological saline soln, **(b)**, (3 + 7, v/v) in high-speed blender ca 5 s. To 50 mL egg yolk emulsion add 10 mL filter sterilized 1% K tellurite soln. Mix and store at 4 ± 1°.

(3) Complete medium.—Add 5 mL warmed enrichment to 95 mL molten basal medium cooled to 45–50°. Mix well, avoiding bubbles, and pour 15–18 mL into sterile 100 × 15 mm petri dishes. Store plates at room temp. (≤25°) for ≤5 days before use. Medium should be densely opaque; do not use nonopaque plates. Dry plates before use by 1 of following methods: (*a*) in convection oven or incubator 30 min at 50° with lids removed and agar surface downward; (*b*) in forced-draft oven or incubator 2 h at 50° with lids on and agar surface upward; (*c*) in incubator 4 h at 35° with lids on and agar surface upward; or (*d*) on laboratory bench 16–18 h at room temp. with lids on and agar surface upward.

(d) *Brain-heart infusion (BHI) broth.*—Dissolve infusion from 200 g calf brain and from 250 g beef heart, 10.0 g proteose peptone or gelysate, 5.0 g NaCl, 2.5 g $Na_2HPO_4 \cdot 12H_2O$, and 2.0 g glucose in 1 L H_2O, heating gently if necessary. Dispense 5 mL portions into 16 × 150 mm test tubes and autoclave 15 min at 121°. Final pH, 7.4 ± 0.2.

(e) *Desiccated coagulase plasma (rabbit) with EDTA.*—Reconstitute according to manufacturer's directions. If not available, reconstitute *desiccated coagulase plasma (rabbit)* and add Na_2H_2EDTA to final concn of 0.1% in reconstituted plasma.

(f) *Butterfield's buffered phosphate diluent.*—*(1) Stock soln.*—Dissolve 34.0 g KH_2PO_4 in 500 mL H_2O, adjust to pH 7.2 with ca 175 mL 1N NaOH, and dil. to 1 L. Store in refrigerator. *(2) Diluent.*—Dil. 1.25 mL stock soln to 1 L with H_2O. Prep. diln blanks with this soln, dispensing enough to allow for losses during autoclaving. Autoclave 15 min at 121°.

Table 3. Collaborative results on comparison of media used in method for counting (log_{10}) *S. aureus* strain F42 in turkey

	Low level				Mid level				High level				Uninoculated			
	TSBS		PTSBS		TSBS		PTSBS		TSBS		PTSBS		TSBS		PTSBS	
Coll.	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
1	2.63	2.32	2.59	2.63	4.63	3.64	4.32	4.18	5.36	4.97	5.63	5.63	<0.48	<0.48	0.56	<0.48
2	2.18	2.63	2.63	2.32	4.36	4.59	3.97	4.32	5.36	5.63	5.97	5.59	<0.48	<0.48	<0.48	0.56
3	2.63	2.63	2.97	2.97	3.87	4.97	4.36	4.36	5.36	5.36	5.97	5.63	<1.48	<1.48	<1.48	<1.48
4	2.36	2.36	2.36	2.36	3.36	4.97	4.18	4.36	6.04	5.66	5.66	5.66	<0.48	<0.48	<0.48	<0.48
5	2.97	2.63	2.63	2.36	3.97	3.64	3.87	4.63	5.97	5.63	5.97	5.36	<0.48	<0.48	<0.48	<0.48
6	2.36	1.96	2.63	2.97	3.64	4.63	4.97	4.36	5.97	5.36	5.97	5.97	<1.48	<1.48	<1.48	<1.48
7	2.18	2.63	2.63	2.88	3.36	4.63	3.64	4.18	4.97	5.38	4.97	4.97	0.79	0.96	<0.48	0.56
8	2.63	2.36	2.97	2.63	4.36	4.59	4.18	3.97	5.38	4.88	5.66	4.88	<0.48	<0.48	<0.48	<0.48
9	2.63	2.36	2.97	1.86	3.64	3.87	3.97	3.97	4.18	4.18	5.38	5.38	<1.48	<1.48	1.55	1.55
10	2.36	2.63	2.63	2.63	3.59	3.97	4.18	3.97	4.97	4.63	5.36	4.97	<0.48	<0.48	<0.48	<0.48
11	0.48[a]	0.48[a]	4.36[a]	4.36[a]	0.48[a]	3.36[b]	4.36	4.36	0.48[a]	0.48[a]	4.36[a]	4.36[a]	0.56	<0.48	4.36	4.81
12	2.63	2.88	3.64	2.88	3.97	3.97	3.97	3.64	5.59	5.36	5.59	5.36	0.48	<0.48	<0.48	<0.48
14	2.36	2.63	2.63	3.36	3.64	3.64	4.36	5.36	5.97	5.36	5.63	5.59	<0.48	1.36	<0.48	3.36
15	2.36	2.18	2.36	2.36	3.36	3.97	3.97	4.36	4.63	4.63	5.88	5.59	<0.48	<0.48	<0.48	0.56
16	1.63	1.97	2.97	2.63	3.36	3.36	4.36	4.63	4.97	5.63	5.36	5.36	<0.48	0.48	0.56	0.48
n_L[c]	14		14		14		15		14		14					
$\bar{x}$	2.43		2.70		3.98		4.22		5.26		5.52					
S_L	0.1973		0.1353		0		0.1544		0.4273		0.2256					
CV_L, %	3.52		2.18		—		1.59		3.53		1.78					
S_o	0.2142		0.3223		0.5406		0.2531		0.2829		0.2333					
CV_o, %	3.83		5.19		5.89		2.60		2.33		1.84					
S_x	0.0729		0.0659		0.0967		0.0553		0.1261		0.0747					
S_r	0.2913		0.3486		0.5406		0.2950		0.5124		0.3245					
CV_r, %	5.20		5.62		5.89		3.04		4.23		2.55					

[a] Statistical outlier by Dixon test (10).

[b] No replicate mating value; not used in statistical computations.

[c] See Table 2, footnote *a*, for definition of terms used in statistical evaluation.

Preparation of Food Homogenate

Aseptically weigh 50 g unthawed food sample into sterile blender jar. Add 450 mL phosphate-buffered diln H_2O and homogenize 2 min at high speed (16 000–18 000 rpm). Use this 1:10 diln to prep. serial dilns from 10^{-2} to 10^{-6} by transferring 10 mL of 1:10 diln to 90 mL diln blank, mixing well with vigorous shaking, and continuing until 10^{-6} is reached.

Most Probable Number Technic

Inoculate 3 tubes of trypticase soy broth with 10% NaCl and 1% sodium pyruvate (**a**) at each test diln with 1 mL aliquots of decimal dilns of sample. Max. diln of sample must be high enough to yield neg. end point. Incubate 48 h at 35°.

Using 3 mm loop, transfer 1 loopful from each growth-pos. tube to dried Baird-Parker medium plates, (**c**)(*3*). Vortex-mix tubes before streaking if growth is visible only on bottom or sides of tubes. Streak so as to obtain isolated colonies. Incubate 48 h at 35–37°.

Interpretation

Colonies of *S. aureus* are typically circular, smooth, convex, moist, 2–3 mm in diam. on uncrowded plates, gray-black to jet-black, frequently with light-colored (off-white) margin, surrounded by opaque zone (ppt), and frequently with outer clear zone; colonies have buttery to gummy consistency when touched with inoculating needle. Occasional non-lipolytic strains may be encountered which have same appearance, except that surrounding opaque and clear zones are absent. Colonies isolated from frozen or desiccated foods which have been stored for extended periods are frequently less black than typical colonies and may have rough appearance and dry texture.

Confirmation Technic

From each plate showing growth, pick ≥1 colony suspected to be *S. aureus*. With sterile needle transfer colonies to tubes contg 0.2 mL BHI broth, (**d**), and to agar slants contg any suitable maintenance medium, e.g., trypticase soy agar, std plate count agar, etc. Incubate BHI culture suspensions and slants 18–24 h at 35°. Retain slant cultures at room temp. for ancillary or repeat tests, in case coagulase test results are questionable.

To BHI cultures add 0.5 mL reconstituted coagulase plasma with EDTA, (**e**), and mix thoroly. Incubate at 35–37° and examine periodically over 6 h interval for clot formation. Any degree of clot formation is considered pos. reaction. Small or poorly organized clots may be observed by gently tipping tube so that liq. portion of reaction mixt. approaches lip of tube; clots will protrude above liq. surface. Coagulase-pos. cultures are considered to be *S. aureus*. Test pos. and neg. controls simultaneously with cultures of unknown coagulase reactivity. Recheck doubtful coagulase test results on BHI cultures which have been incubated at 35–37° for >18 but ≤48 h.

Report most probable number (MPN) of *S. aureus*/g from tables of MPN values. Table **46:01.**

Results

All reported data were checked for correct MPN calculations. The data were transformed by base 10 logarithms before analysis to obtain approximate normality of statistical distribution. Separate analyses of variance were compiled for TSBS and PTSBS at low, middle, and high levels for each of the 3 foods. This enabled separate estimates of means, variation between laboratories, and variation within laboratories. The data and summary statistics are given in Tables 2–4.

PTSBS was successful in significantly ($\alpha = 0.05$) increasing enumeration of *S. aureus* in all but one comparison. Recovery of low level *S. aureus* from tuna salad in PTSBS was not significantly different ($\alpha = 0.10$) from recovery in TSBS. The MPN technique using PTSBS also exhibited higher overall repeatability and reproducibility with samples inoculated at all 3 population levels. This can be seen by comparing the standard deviations and coefficients of variations reflecting the repeatability and reproducibility of results with the 2 media (Tables 2–4).

Despite the efforts of the Associate Referee to obtain products without an indigenous *S. aureus* population, the ground turkey was naturally contaminated with low levels of *S. aureus*. This was evidenced by the recovery of viable *S. aureus* in 16 of 30 uninoculated samples tested. However, the naturally occurring *S. aureus* was found in 13 of 16 turkey

Table 4. Collaborative results on comparison of media used in method for counting (log_{10}) *S. aureus* strain 493 in tuna salad

	Low level				Mid level				High level				Uninoculated			
	TSBS		PTSBS		TSBS		PTSBS		TSBS		PTSBS		TSBS		PTSBS	
Coll.	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
1	3.64	2.97	3.17	3.36	4.97	3.64	4.97	4.36	6.66	6.74	6.66	5.97	<0.48	<0.48	<0.48	<0.48
2	2.36	2.36	2.63	3.17	—[a]	4.63[b]	—[a]	4.88[b]	5.36	5.36	6.66	6.18	—[a]	<0.48	—[a]	<0.48
3	2.63	2.97	2.97	3.36	4.63	4.63	5.36	4.97	5.88	6.38	6.18	5.97	<1.48	<1.48	<1.48	<1.48
4	2.97	3.36	3.36	2.36	4.32	4.36	5.36	4.32	5.63	5.97	5.63	5.97	<0.48	<0.48	<0.48	<0.48
5	2.63	2.63	3.36	3.64	4.36	3.97	4.36	4.36	5.36	5.36	6.46	5.63	<0.48	<0.48	2.63	<0.48
6	2.97	3.21	2.63	2.97	3.97	4.36	4.97	4.36	5.36	5.36	6.18	6.38	<1.48	<1.48	<1.48	<1.48
7	2.63	2.63	2.97	2.63	3.97	4.08	4.36	3.97	4.97	5.38	5.38	5.38	<0.48	<0.56	<0.48	<0.48
8	2.97	3.64	2.97	3.64	4.97	3.97	4.97	4.88	5.97	6.38	5.63	6.38	<0.48	<0.48	<0.48	<0.48
9	2.36	2.63	2.36	2.63	3.64	3.97	4.63	4.36	4.32	4.97	5.66	5.66	<1.48	<1.48	<1.48	<1.48
10	2.97	3.36	2.97	3.36	4.63	4.88	4.63	4.63	5.97	5.63	5.63	5.97	<0.48	<0.48	<0.48	<0.48
11	3.17	2.97	4.36[c]	4.36[c]	3.97	4.63	4.63	4.97	4.97	5.63	5.63	5.97	<0.48	<0.48	4.36	4.36
	2.88	3.36	3.36	3.17	4.63	4.36	4.63	4.36	5.97	5.36	6.38	5.63	<0.48	<0.48	<0.48	<0.48
14	2.97	2.97	3.64	2.63	3.36	4.36	3.64	3.97	5.63	5.63	6.66	6.38	<0.48	<0.48	<0.48	<0.48
15	2.36	2.36	2.36	2.36	3.36	3.36	3.64	3.36	4.63	4.97	4.63	5.36	<0.48	<0.48	<0.48	<0.48
16	2.97	2.63	2.97	2.63	4.63	4.36	5.18	4.36	5.63	5.63	6.38	6.38	<0.48	<0.48	<0.48	<0.48
n_L[d]	15		14		14		14		15		15					
$\bar{x}$	2.88		2.99		4.23		4.54		5.54		5.97					
S_L	0.2749		0.2006		0.2252		0.3827		0.4167		0.3324					
CV_L, %	4.14		2.918		2.31		3.66		3.27		2.42					
S_o	0.2465		0.3589		0.4164		0.3314		0.3040		0.3443					
CV_o, %	3.71		5.22		4.28		3.17		2.38		2.51					
S_r	0.0841		0.0773		0.0891		0.1200		0.1210		0.1064					
S_R	0.3693		0.4094		0.4714		0.5063		0.5159		0.4785					
CV_R, %	5.56		5.95		4.85		4.85		4.04		3.48					

[a] Laboratory accident; value not reported.
[b] No replicate mating value; not used in statistical computations.
[c] Statistical outlier by Dixon test (10).
[d] See Table 2, footnote *a*, for definition of terms used in statistical evaluation.

samples to be lower than the low level inoculum. Since the same food homogenate was used to compare the 2 media, the natural contamination should not have biased the results in favor of one medium over the other, and a comparison of the inoculated samples could be made.

One laboratory (9) reported that milk samples were analyzed a week later than instructed. Data received from this collaborator were not found to be outliers and therefore the data were not discarded. One laboratory (13) did not analyze samples of tuna salad and ground turkey.

Enumeration of both stressed and nonstressed *S. aureus* cells at each inoculum level was increased when pyruvate was added to TSBS. However, a greater increase in recovery was found with stressed cells than with nonstressed cells. Although this may be due to strain and product variations as well as stress factors, the addition of pyruvate appears especially useful for recovery when heat-stressed *S. aureus* cells are present in foods.

Recommendation

It is recommended that the official final action method for *Staphylococcus aureus* in foods, **46.062,** be replaced with the MPN method using pyruvate-amended medium.

Acknowledgments

The authors thank the following microbiologists who took part in this study:

N. Bisciello, Food and Drug Administration, New York, NY
R. G. Bryant and J. Jarvis, Dept of Health Services, Berkeley, CA
A. Burger, Consolidated Laboratory Services, Richmond, VA
J. C. DeRamon, FDA, San Francisco, CA
L. Estela, FDA, Denver, CO
D. Evanson, Silliker Laboratories, Chicago Heights, IL
D. Y. C. Fung and C. C. S. Lin, Kansas State University, Manhattan, KS
S. Huppler and R. Reuter, Minnesota Dept of Agriculture, St. Paul, MN
K. Kallander, FDA, Minneapolis, MN
C. R. McKee, FDA, Dallas, TX
M. Pratt, U.S. Dept of Agriculture, St. Louis, MO
J. Tom, FDA, New York, NY
D. Waldrop, U.S. Dept of Agriculture, Athens, GA
W. D. Ware, FDA, Cincinnati, OH
H. Willey, Ohio Dept of Agriculture, Reynoldsburg, OH

Appreciation is extended to Stephen W. Butler, Division of Mathematics, FDA, Washington, DC, for statistical assistance.

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DRUGS

Liquid Chromatographic Determination of Penicillin V Potassium in Tablets and Powders for Oral Solution

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A reverse-phase liquid chromatographic method is described for the assay of penicillin V potassium in tablets and powders for oral solution. Under isocratic conditions, the combined use of an octadecylsilane column, with a mobile phase composed of acetonitrile–methanol–0.01M monobasic potassium phosphate (21 + 4 + 75, v/v), and photometric detection at 225 nm, separated penicillin V potassium from excipients, related compounds, and degradation products. Sulfadimethoxine was used as an internal standard. Detector responses were linearly related to concentrations of penicillin V over the range 25–225 μg/mL (r = 0.99997). Standard addition recoveries ranged from 98.8 to 99.9% (mean 99.5%, n = 8) for tablets and from 97.9 to 101.6% (mean 99.8%, n = 8) for powders for oral solution. The liquid chromatographic assay results were compared with those obtained by the official iodometric titration method. The proposed method is simple, selective, stability-indicating, and free from interference by excipients and degradation products.

The official methods for the quantitative determination of penicillin V potassium in oral dosage forms are based on microbiological and titrimetric techniques (1). The microbiological assay is an agar diffusion method which involves slow and tedious preparative steps, and which often yields unreliable results (2, 3). The same method is also susceptible to inaccuracies because of its inability to differentiate among related penicillins. The iodometric titration method is a relatively rapid means of determining the potency of penicillins. However, this procedure is also nonspecific; furthermore, the end point of the titration is affected not only by the consumption of iodine by substances other than penicillin (4) but also by the concentration of iodide in the titration medium and the pH of the medium (5). In view of the above deficiencies and the relative instability of the penicillin molecule, we decided to develop a sensitive, selective reverse-phase liquid chromatographic (RP-LC) method for the assay of penicillin V potassium in dosage forms. This method represents a modification of a qualitative liquid chromatographic procedure previously reported by this laboratory (6) that permitted both the separation and identification of selected penicillins, even in the presence of each other. The method presented here is accurate, selective, and stability-indicating.

Experimental

Apparatus

(a) *Liquid chromatograph.*—Model 510 solvent pump, Model 481 variable wavelength detector, Model U6K injector valve, and Model 730 integrating recorder (Waters Associates, Milford, MA 01757).

(b) *Column.*—Stainless steel, 30 cm × 3.9 mm id, prepacked with 10 μm particle size μBondapak C_{18} (Waters Associates) preceded by a 40 mm Co-Pell ODS LC guard column (Whatman Inc., Clifton, NJ 07014).

Received November 14, 1985. Accepted May 7, 1986.
Presented in part at the 98th AOAC Annual International Meeting, Oct. 28–Nov. 2, 1984, Washington, DC.

Reagents

(a) *Solvents.*—LC grade acetonitrile and methanol.

(b) *Potassium dihydrogen phosphate solution.*—0.01M KH_2PO_4, 1.361 g/L.

(c) *Mobile phase.*—Acetonitrile–methanol–0.01M KH_2PO_4 (21 + 4 + 75, v/v).

(d) *Penicillin V potassium.*—USP reference standard.

(e) *Internal standard solution (IS).*—Dissolve USP Sulfadimethoxine Reference Standard in 50% acetonitrile–water to obtain a concentration of ca 0.5 mg/mL.

(f) *Standard preparation.*—Accurately weigh ca 11 mg penicillin V potassium into a 100 mL volumetric flask, add 15.0 mL IS, dilute to volume with water, and mix.

Sample Preparation

For tablets, prepare powdered sample composite from at least 20 tablets. For powders for oral solution, reconstitute formulation in water according to manufacturer's instruction. Transfer quantity of powdered tablet composite or bulk material, or volume of oral solution, equivalent to 110 mg penicillin V potassium, to a 100 mL volumetric flask, dilute to volume with water, and mix. Filter and transfer 10.0 mL filtrate to another 100 mL volumetric flask, add 15.0 mL IS, and dilute to volume with water.

Table 1. Recovery of penicillin V potassium from commercial tablet composites

Mfr.	Added, mg	Found, mg	Rec., %
250 mg/tablet			
A	5.418	5.396	99.6
B	5.340	5.313	99.5
C	5.340	5.335	99.9
D	5.340	5.303	99.3
Mean	5.360	5.337	99.6
500 mg/tablet			
E	5.418	5.396	99.6
F	5.418	5.407	99.8
G	5.340	5.276	98.8
H	5.340	5.319	99.6
Mean	5.379	5.350	99.5

Table 2. Recovery of penicillin V potassium from commercial powders for oral solution

Mfr.	Added, mg	Found, mg	Rec., %
125 mg/5 mL			
A	5.427	5.514	101.6
B	5.452	5.463	100.2
D	5.427	5.313	97.9
E	5.427	5.449	100.4
I	5.427	5.345	98.5
J	5.452	5.409	99.2
Mean	5.435	5.416	99.6
250 mg/5 mL			
G	5.452	5.422	99.4
H	5.452	5.496	100.8
Mean	5.452	5.459	100.1

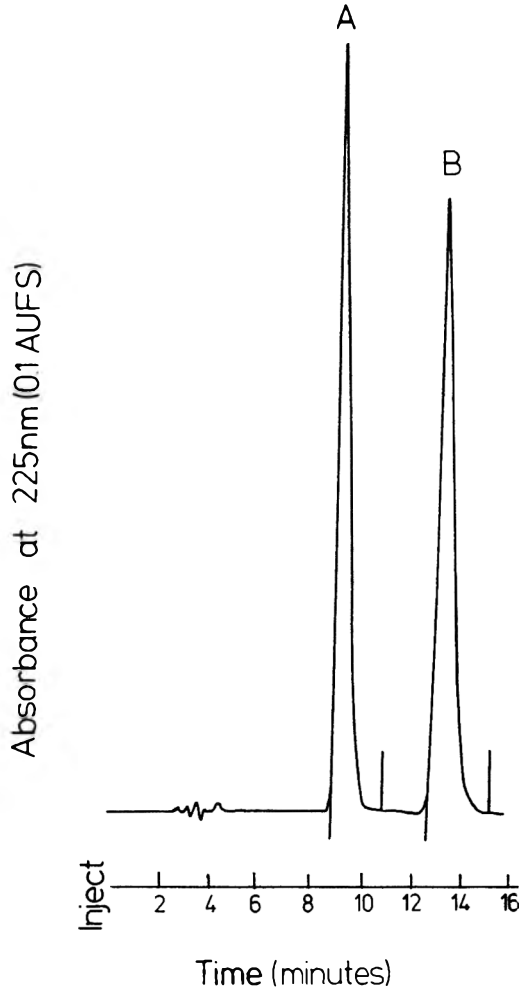

Figure 1. Typical chromatogram of a mixture of (A) USP penicillin V potassium and (B) sulfadimethoxine, the internal standard. Conditions: column = μBondapak C_{18}; mobile phase = acetonitrile–methanol–0.01M potassium phosphate, monobasic, pH 4.7 (21 + 4 + 75, v/v); flow rate = 1 mL/min; chart speed = 0.5 cm/min.

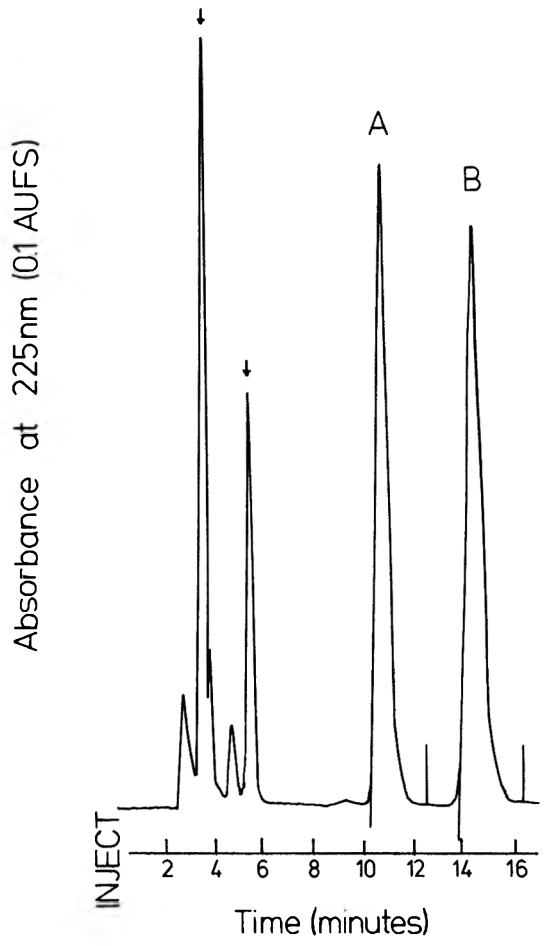

Figure 2. Typical chromatogram of a powder for oral solution. (A) Penicillin V potassium and (B) sulfadimethoxine, the internal standard. Arrows indicate excipients and/or degradates. Conditions: as for Figure 1.

Assay

Inject 20 μL each of standard preparation and sample preparation into chromatograph while mobile phase is flowing at rate of 1 mL/min; detector is set at 225 nm, 0.1 AUFS; and chart speed is 0.5 cm/min.

Calculations

Obtain quantity of penicillin V (free acid) in the sample as follows: mg/tab. = $(R_u/R_s) \times C \times D \times (T/W) \times (1/F)$, or mg/5 mL oral solution = $5 \times (R_u/R_s) \times C \times D \times (V/G) \times (1/F)$, in which R_u and R_s are ratios of peak area of penicillin V potassium to peak area of IS for sample preparation and standard preparation, respectively; C is potency of standard preparation of penicillin V, in units/mL; D is sample dilution factor; T is g average tablet weight; W is g sample composite taken for analysis; V is mL total volume of oral solution; G is mL oral solution taken for analysis; and F is equivalency factor of penicillin V, 1600 units/mg.

Results and Discussion

The results of standard recovery studies of penicillin V potassium from sample composites of commercial tablets

Table 3. Comparison of potency determination of penicillin V potassium in tablets by official iodometric titration and LC methods

Mfr.	Tablet type	Declared, mg/tab.	Found, % of decl. CFR(A)	Found, % of decl. LC(B)	Difference A − B, % of decl.
A	uncoated	250	99.7	99.1	0.6
B	film-coated	250	107.1	105.5	1.6
C	uncoated	250	109.4	107.0	2.4
D	uncoated	250	110.5	106.9	3.6
E	uncoated	500	104.2	104.5	−0.3
F	uncoated	500	98.1	94.9	3.2
G	uncoated	500	103.7	102.5	1.2
H	film-coated	500	98.2	98.4	−0.2
Mean			103.9	102.4	1.50
SD			4.90	4.45	1.47
CV, %			4.72	4.35	—

Table 4. Comparison of potency determination of penicillin V potassium in powders for oral solution by official iodometric titration and LC methods

Mfr.	Declared, mg/5 mL	Found, % of decl. CFR(A)	Found, % of decl. LC(B)	Difference A − B, % of decl.
A	125	110.6	101.1	9.5
B	125	101.4	100.4	1.0
D	125	108.4	107.5	0.9
E	125	115.9	116.0	−0.1
I	125	101.1	106.3	−5.2
J	125	115.1	115.8	−0.7
G	250	105.5	99.0	6.5
H	250	104.2	104.2	0
Mean		107.8	106.3	1.49
SD		5.75	6.60	4.54
CV, %		5.33	6.21	—

and powders for oral solution are shown in Tables 1 and 2, respectively. The average recovery value was 99.5% for tablets and 99.8% for powders for oral solution. These data indicate that the proposed RP-LC method is relatively unaffected by the sample matrix.

Detector response ratios were linearly related to concentrations of penicillin V over the range 25–225 μg/mL (correlation coefficient, $r = 0.99997$). The coefficient of variation for 5 replicate injections of a standard preparation was less than 0.1%. A chromatogram of penicillin V potassium is shown in Figure 1. The retention time was within 9–11 min, and the tailing factor was 1.4. The resolution factor (R) of the standard and IS, sulfadimethoxine, was greater than 4.

The assay results for 16 commercial samples of penicillin V potassium tablets and powders for oral solution obtained by the official (CFR) iodometric titration method and the proposed method are presented in Tables 3 and 4, respectively. At the 95% confidence level, no significant intermethod differences were noted for the assay results for powders for oral solutions (Table 4). By contrast, the assay results for tablet composites (Table 3) obtained by the official method were statistically significantly higher than those obtained by the proposed chromatographic method. These higher results are not unexpected, since the official iodometric method is nonselective, and, as pointed out earlier in the introduction, the end point of the titration is affected by other variables (4, 5).

The proposed assay method for penicillin V potassium offers improved selectivity over the official (CFR) method. Penicillin V elutes at an intermediate rate and is separated from other major penicillins, including ampicillin, amoxicillin, methicillin, oxacillin, penicillin G, nafcillin, dicloxacillin, and cloxacillin. In addition, the peaks for penicillin V and the IS were well resolved from peaks belonging to potentially important interferences such as excipients and degradation products, as demonstrated by the chromatogram shown in Figure 2. When solutions of penicillin V potassium were experimentally degraded with the aid of base or acid, the resulting mixtures yielded chromatograms containing additional peaks, none of which interfered with the interpretation and measurement of the chromatographic peaks for the analyte and IS (approximate retention times 10 and 14 min, respectively), as shown in Figure 3.

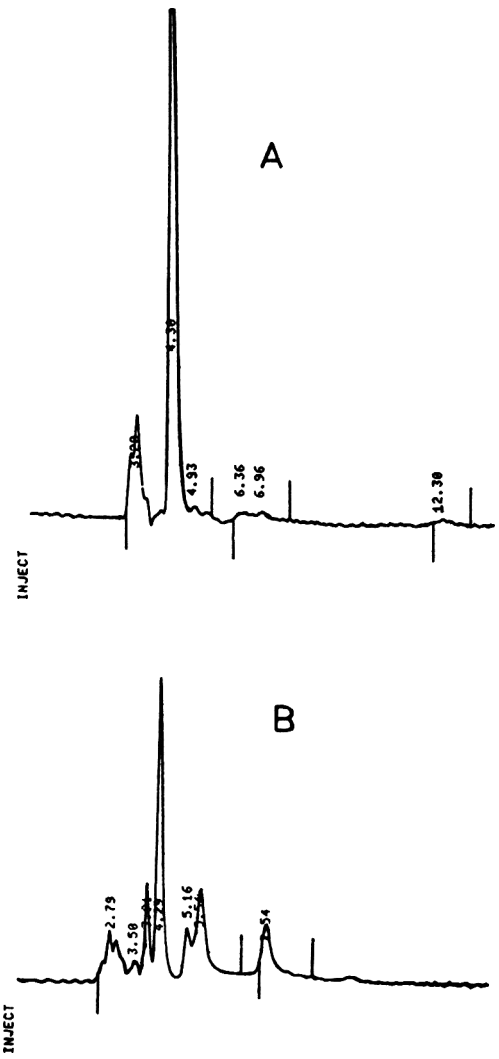

Figure 3. Typical chromatograms of completely degraded samples of penicillin V potassium. A, acid degradation; B, base degradation. Conditions: as for Figure 1. Numbers indicate retention times in minutes. Degradation products were not identified.

Conclusion

This study demonstrated the applicability of the proposed LC method for the quantitative determination of penicillin V potassium in commercial tablets and powders for oral solution. Advantages of the LC method over the official method include greater selectivity, less interference by excipients and degradation products, and ability to provide information on drug stability.

Acknowledgments

The author thanks Cesar A. Lau-Cam, Science Advisor, Food and Drug Administration, New York Regional Laboratory, and Professor of Pharmaceutical Sciences, St. John's University, College of Pharmacy and Allied Health Professions, Jamaica, NY, for his helpful advice and assistance in the preparation of this paper, and Robert W. Roos, Food and Drug Administration, for his useful suggestions.

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Ultraviolet Spectrophotometric Determination of Hydralazine Hydrochloride in Tablets Following Derivatization with Nitrite

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Routine use of the USP XXI spectrophotometric method for the content uniformity determination of hydralazine hydrochloride tablets has shown that tablet excipients can significantly alter the spectral characteristics of the drug and thus cause inaccurate assay values to be obtained. Because of this problem, a simple and reliable alternative spectrophotometric assay method, based on the conversion of hydralazine to tetrazolo [5,1-α]phthalazine with nitrite ions under acidic conditions, was developed. The derivative showed an absorption maximum at about 274 nm and obeyed Beer's law over the concentration range 4-40 μg/mL. Mean recoveries of hydralazine hydrochloride added to commercial coated and uncoated tablets were 101.0% (n = 10) and 100.8% (n = 8), respectively. The proposed method was found suitable for the assay not only of individual tablets but also of tablet composites.

Hydralazine hydrochloride, 1(2*H*)-phthalazinone hydrazone monohydrochloride, is a phthalazine derivative which, by exerting a direct relaxing effect on vascular smooth muscles, can lower both the arterial blood pressure and peripheral vascular resistance. Although clinically used since the early 1950s, only in recent years has this drug become widely prescribed for the long-term management of essential hypertension.

Various spectrophotometric methods described for the quantitative analysis of hydralazine have entailed the formation of ultraviolet (UV)-absorbing derivatives or colored products upon condensation of hydralazine with aldehydes (1, 2) or complexation with 9-chloroacridine (3), tetrazolium blue (4), ninhydrin (5), and 1,10-phenanthroline in the presence of ferrous ions (6). These methods have drawbacks such as the need for elevated temperature for the derivatization reaction, close control of the experimental conditions (i.e, concentration, pH, and reaction time), and instability of the reaction product. The official method for the determination of the content uniformity of hydralazine hydrochloride tablets is a simpler ultraviolet spectrophotometric one (7). However, when this method was used to analyze commercial coated and uncoated tablets, differences were observed between the spectra of a number of samples and the spectrum of a standard preparation. The differences were significant enough to suggest that the official method was not entirely reliable for regulatory work.

This paper describes a simple, accurate, and precise spectrophotometric assay method for hydralazine hydrochloride tablets which is based on the conversion of hydralazine to tetrazolo [5,1-α]phthalazine by nitrite ions (8, 9) (Figure 1).

This reaction was first applied by Jack et al. (8) for the gas chromatographic determination of hydralazine in plasma and was subsequently used by Noda et al. (9) for the liquid chromatographic measurement of nitrite ions in biological fluids, foods, and ambient waters. The proposed method circumvents the problems associated with the other spectroscopic assay methods and with the official content uniformity method in particular.

Received January 31, 1985. Resubmitted January 31, 1986. Accepted March 30, 1986.

Presented in part at the 37th National Meeting of the American Pharmaceutical Association-Academy of Pharmaceutical Sciences, Oct. 28–Nov. 1, 1984, Philadelphia, PA.

Experimental

Apparatus and Reagents

(a) *Spectrophotometer.*—Cary 219 UV-visible recording spectrophotometer (Varian Instrument Group, Palo Alto, CA).

(b) *Hydralazine hydrochloride.*—USP Reference Standard, dried over phosphorus pentoxide under vacuum.

(c) *Sodium nitrite solution.*—1%, freshly prepared.

(d) *Hydrochloric acid solution.*—0.1N.

(e) *Hydralazine hydrochloride solution.*—Prepared in 0.1N HCl to contain 1 mg/mL.

Samples

Obtained from commercial sources and represented by 25 and 50 mg uncoated tablets (6 manufacturers) and 10, 25, 50, and 100 mg coated tablets (4 manufacturers).

Sample Preparation

Weigh and powder not less than 20 tablets. Transfer a portion of powder or a crushed tablet to volumetric flask of volume suitable to obtain final hydralazine hydrochloride concentration of ca 0.1 mg/mL. Dilute to volume with 0.1N HCl, mix well, and filter, discarding first 20 mL filtrate. Transfer 20 mL filtrate to a 100 mL volumetric flask, add 1 mL sodium nitrite solution, mix, and heat on steam bath ca 1 h. Cool solution to room temperature, dilute to volume with water, and mix.

Standard Preparation

Prepare solution of hydralazine hydrochloride in 0.1N HCl to contain ca 0.1 mg/mL. Pipet 20 mL of this solution into a 100 mL volumetric flask and proceed as in *Sample Preparation,* beginning, "add 1 mL sodium nitrite solution . . ."

Linearity Study

Prepare stock solution of hydralazine in 0.1N HCl to contain ca 0.2 mg/mL. Pipet aliquots of this solution, representing amounts of hydralazine hydrochloride ranging from 0.4 to 4.0 mg, into individual 100 mL volumetric flasks. To each flask add enough 0.1N HCl to obtain total volume of ca 20 mL. Proceed as in *Sample Preparation,* beginning, "add 1 mL sodium nitrite solution . . ."

Recovery Study

Transfer portion of powdered tablet composite, equivalent to ca one-half the amount of hydralazine hydrochloride found in 1 tablet, to volumetric flask of volume suitable to obtain

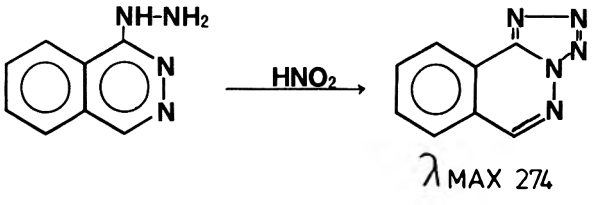

Figure 1. Chemical structures of (left) hydralazine and (right) tetrazolo [5,1-α]phthalazine.

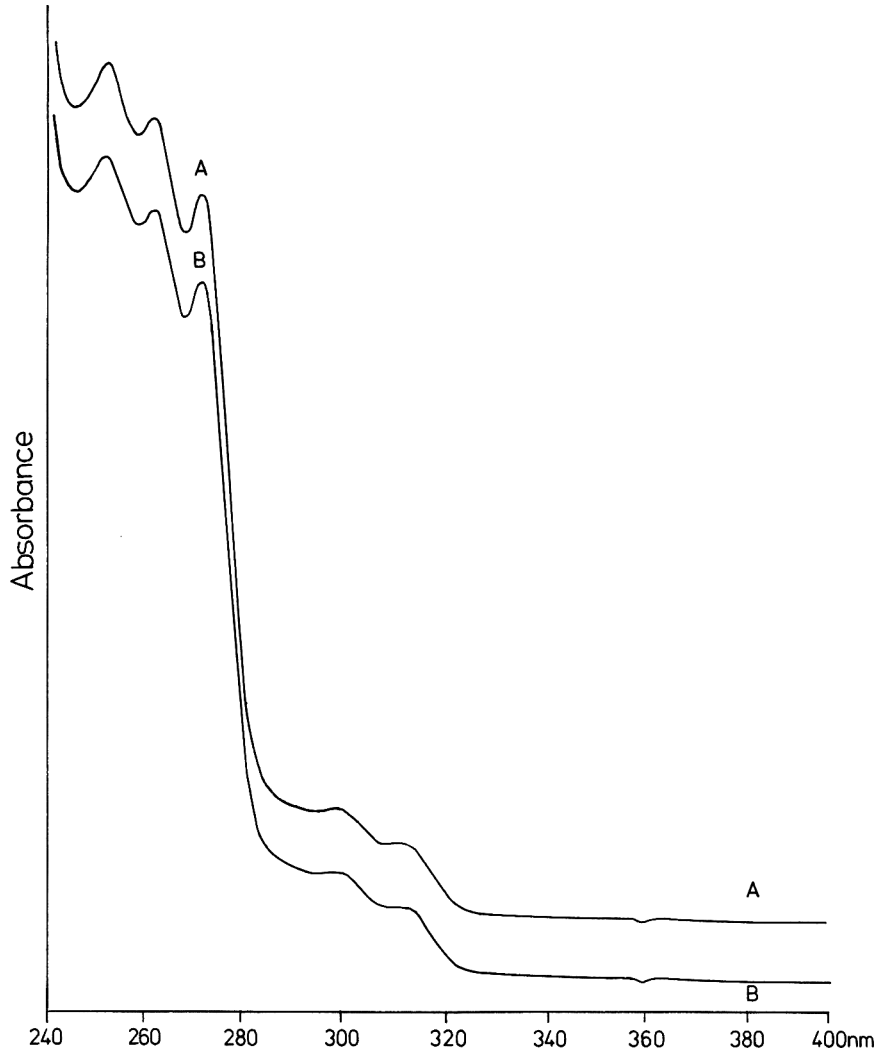

Figure 2. Comparison of typical UV spectra of (A) standard and (B) sample preparations.

final concentration of ca 0.05 mg/mL. Add volume of hydralazine hydrochloride solution equivalent to one-half the amount of drug found in 1 tablet. Proceed as in *Sample Preparation,* beginning, "Dilute to volume with 0.1N HCl."

Assay

Using suitable spectrophotometer, concomitantly determine absorbances of standard preparation and sample preparation in 1 cm quartz cells, at wavelength of maximum absorbance at ca 274 nm, using blank prepared as in *Sample Preparation.* Calculate quantity of hydralazine hydrochloride, in mg/tablet, from formula: $(A_u/A_s) \times C \times D\ (T/W)$, where A_u = absorbance of sample preparation; A_s = absorbance of standard preparation; C = concentration of standard preparation, in mg/mL; D = sample dilution factor; T = mg average tablet weight; and W = mg sample taken.

Results and Discussion

Characterization of Tetrazolo [5,1-α]Phthalazine

The derivative was isolated from the reaction mixture by crystallization and characterized by spectroscopic methods. The IR spectrum exhibited major bands at 3030, 1620, 1588, and 760 cm^{-1}, and the absence of N—H stretching vibrations was noted. The ^{1}H-NMR spectrum in dimethyl sulfoxide showed resonance signals for aromatic protons (doublets at δ8.44 and 8.68 ppm, and multiplet at δ8.20 ppm) and imine proton adjacent to a phenyl ring (singlet at δ9.46 ppm). The negative CI mass spectrum contained a molecular ion peak at m/z 171 and peaks at m/z 143 and 115, corresponding to the successive losses of N-containing groups. The DSC thermogram indicated a sharp melting at 210–211°C, with subsequent decomposition at about 212°C.

Maximum yields of derivative were obtained at pH values below 2.0 and at temperatures of 70–90°C. By monitoring the intensity of the UV absorption at 274 nm, we noticed that rapid conversion started to occur after 5 min of heating and was essentially complete after 30 min. Concentrations of nitrite ions between 1 and 3% yielded approximately the same absorbance values at 274 nm. Therefore, a concentration of 1% was adopted in the final method. The UV spectrum of the derivative exhibited absorption maxima at about 256, 265, and 274 nm, and absorption minima at about 250, 261, and 270 nm (Figure 2). The absorption peak at 274 nm (ϵ = 5676) was selected for quantitative work, since interference by nitrite ions was found to be significant at lower wavelengths.

Linearity and Recovery Studies

Absorbances at 274 nm were linearly related to concentrations of hydralazine hydrochloride ranging from 4 to 40 μg/mL (r = 0.99994). The results of recovery experiments for hydralazine hydrochloride added to sample composites

Table 1. Recovery of hydralazine hydrochloride from commercial tablet composites

Mfr.	Declared, mg/tab.	Added, mg	Found, mg	Rec., %
		Uncoated tablets		
A	25	12.52	12.37	98.8
	50	25.04	25.14	100.4
B	50	25.04	25.79	103.0
C	50	25.04	25.42	101.5
D	25	12.52	12.65	101.0
	50	25.04	24.96	99.7
E	50	25.04	25.49	101.8
F	25	12.52	12.54	100.2
Av.				100.8
		Coated tablets		
G	25	12.52	12.38	98.9
	50	25.04	25.79	103.0
H	25	12.52	12.66	101.1
	50	25.04	24.69	98.6
I	25	12.52	12.46	99.5
	50	25.04	25.64	102.4
J	10	5.01	5.19	103.6
	25	12.52	12.81	102.3
	50	25.04	25.74	102.8
	100	50.08	49.18	98.2
Av.				101.0

of commercial coated and uncoated tables are given in Table 1. Recoveries from 25 and 50 mg uncoated tablets ranged from 98.8 to 101.0% (mean = 100%, n = 3) and from 99.7 to 103.0% (mean = 101.3%, n = 5), respectively. Recoveries from 10, 25, 50, and 100 mg coated tablets ranged from 98.2 to 103.6% (mean = 101.0%, n = 10).

Comparison of Proposed Method with Compendial Methods

The official method for the content uniformity determination of hydralazine hydrochloride tablets consists of dissolving the tablet in aqueous methanol and spectrophotometrically measuring the absorption of the solution at about 260 nm (7). During routine analyses of individual coated and uncoated tablets of hydralazine hydrochloride from various manufacturers, we noticed that the UV absorption spectra of a number of the samples differed markedly, both in shapes and intensities, from that of a standard solution. These differences were quite significant in the spectral region 220–270 nm, which includes the recommended determinative wavelength. In addition to a weakening in absorption at about 260 nm, these samples yielded a shoulder at about 270 nm (Figure 3). At this point it seemed reasonable to assume that a determination made at about 260 nm would yield a falsely lower assay value.

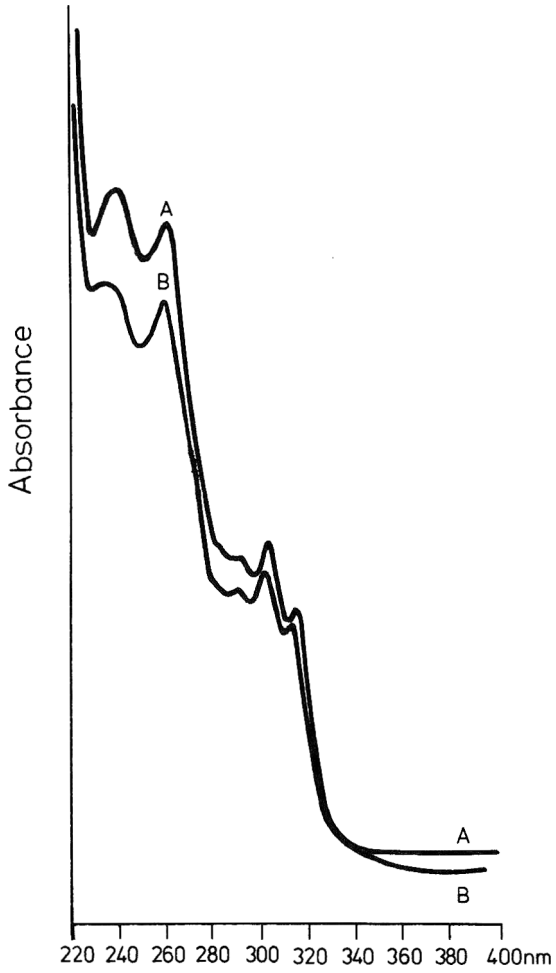

Figure 3. Comparison of typical UV spectra of (A) standard and (B) sample solutions prepared as specified by the USP XXI content uniformity procedure.

Normally, analytical error due to the USP special procedure (10) is "corrected" by comparing the results obtained by this method on composite samples with those obtained

Table 2. Assay results for individual 50 mg uncoated tablets by proposed and USP XXI content uniformity methods

	Found, % of declared		
Tablet No.	Proposed method	Tablet No.	USP XXI method
1	91.8	11	104.7
2	99.8	12	84.9
3	119.4	13	98.4
4	90.7	14	105.7
5	112.3	15	96.0
6	92.8	16	90.4
7	105.5	17	118.5
8	111.3	18	78.6
9	93.3	19	90.4
10	92.8	20	83.8
Av.	101.0		95.1
SD	10.42		12.07
CV, %	10.32		12.70
USP limits, %		85.0–115.0	

Table 3. Assay results for hydralazine hydrochloride tablet composites by proposed and USP XXI content uniformity methods

			Found, % of declared		
Mfr.	Tablet type	Declared, mg/tab.	Proposed method (B)	USP XXI method (A)	(B) − (A), % of decl.
B	uncoated	50	98.6	95.4	3.2
D	uncoated	25	101.4	98.8	2.6
F	uncoated	25	99.8	96.0	3.8
G	coated	25	98.5	94.2	4.3
H	coated	25	96.4	91.4	5.0
I	coated	50	100.7	97.3	3.4
J	coated	25	99.4	96.6	2.8
J	coated	50	100.0	96.8	3.2
Av.			99.4	95.8	3.5
SD			1.54	2.24	0.80
CV, %			1.55	2.33	—

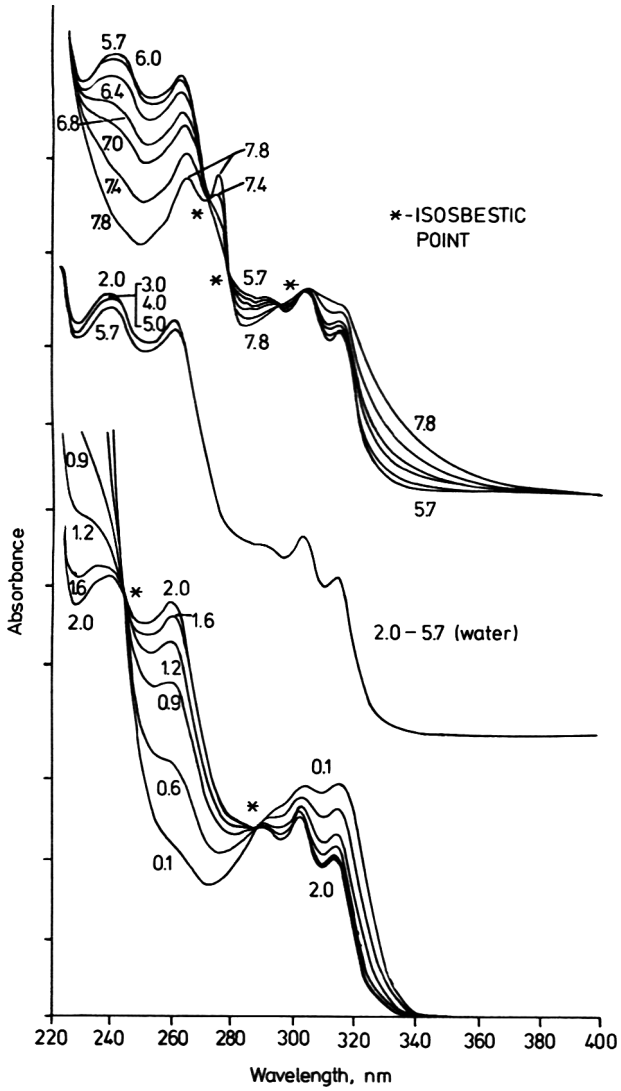

Figure 4. Spectra of aqueous solutions of hydralazine hydrochloride (10 μg/mL) at various pH values. Numbers indicate pH values.

by the compendial assay method. In the case of hydralazine hydrochloride tablets, however, the degree of distortion of the absorption spectra for some samples was of such a magnitude as to render the correction method impractical. The assumption that a lower assay value would be obtained by the compendial content uniformity method than by the proposed method is corroborated by the results shown in Tables 2 and 3. Table 2 presents individual tablet assay results for 2 sets of 50 mg uncoated tablets from the same batch, one of which was analyzed by the official method and the other by the proposed method. On the average, the compendial method yielded a result that was 5.9% lower (± 10.6%, at the 95% confidence level) than that obtained by the proposed method. This downward trend is also noted in Table 3, which compares the assay results for sample composites of tablets from various manufacturers as obtained by the official content uniformity method and the proposed method. The former method yielded values significantly lower (95% confidence level) than those obtained by the latter method. To demonstrate the reliability of the proposed method, the same tablet sample composite was analyzed by both the proposed method and the iodometric titration method of USP XX (11). The results presented in Table 4 indicate statistically significant agreement between the methods.

Table 4. Assay results for hydralazine hydrochloride tablet composites by proposed and USP XX (titrimetric) assay methods

Mfr.	Declared, mg/tab.	Found, % of declared: Proposed method (B)	Found, % of declared: USP XX method (A)	(B) − (A), % of decl.
		Uncoated tablets		
A	25	105.0	102.6	2.4
	50	103.2	100.6	2.6
B	50	98.6	98.4	0.2
C	25	95.8	96.3	−0.5
	50	96.2	97.5	−1.3
D	25	101.4	103.0	−1.6
	50	100.4	101.8	−1.4
E	50	98.0	97.2	0.8
F	25	99.8	100.2	−0.4
Av.		99.8	99.7	0.09
SD		3.1	2.48	1.57
CV, %		3.1	2.48	—
		Coated tablets		
G	25	98.5	97.8	0.7
	50	100.8	100.2	0.6
H	25	96.4	95.4	1.0
	50	95.6	95.4	0.2
I	25	99.8	100.2	−0.4
	50	100.7	99.9	0.8
J	10	108.0	107.6	0.4
	25	99.4	99.2	0.2
	50	99.0	98.5	0.5
	100	100.0	99.3	0.7
Av.		99.8	99.4	0.47
SD		3.35	3.40	0.40
CV, %		3.35	3.42	—

Effect of pH on the Ultraviolet Absorption Spectrum of Hydralazine

The absorption spectra of hydralazine hydrochloride solutions were found to be influenced by pH, as shown in Figure 4. Spectral changes were more pronounced around pH values of 0.5 and 6.9, which correspond to the two pK_a values of the drug (12). This behavior is probably related to ionization changes undergone by the hydralazine molecule when in solution. Since in solution hydralazine hydrochloride can exist as an equilibrium of 3 interconvertible forms, i.e., the monoprotonated (monohydrochloride), diprotonated (dihydrochloride), and un-ionized (neutral) forms, each with unique absorption characteristics, any change in pH will affect their individual concentrations and therefore will alter the absorption spectrum of the drug. The pH-related spectral sensitivity noted during the performance of the compendial method for content uniformity determination of hydralazine hydrochloride tablets appears to be associated with excipients present in certain brands of tablets. These excipients tended to raise the pH of the sample solutions to values in the vicinity of the higher pK_a. Interestingly, absorbance values remained constant (isosbestic points) at about 240, 270, and 290 nm over limited pH ranges (Figure 4). The use of a buffer solution was initially considered for the UV spectrophotometric determination of hydralazine. However, because of evidence of drug decomposition in buffer solutions such as potassium phosphate, the derivatization procedure prevailed.

The proposed method avoids the problems related to the effects of pH on the ionization states of hydralazine, since the absorption spectrum of its phthalazine derivative will

remain invariant to wide changes in hydrogen ion concentration. Consequently, it is a more reliable method for content uniformity determinations than the compendial method, as well as being equally suited for the analysis of composited tablet samples.

Acknowledgments

The author thanks Cesar A. Lau-Cam, Science Advisor, Food and Drug Administration, New York Regional Laboratory, and Professor of Pharmaceutical Sciences, St. John's University, College of Pharmacy and Allied Health Professions, Jamaica, NY, for his helpful advice and assistance in the preparation of this paper. Appreciation is also extended to the following colleagues at the Food and Drug Administration: Marten E. Woodhouse, Deborah J. Genova, Yoko H. Doucette, Gary Lehr, Glenn Petzinger, and John W. Turczan.

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PESTICIDE FORMULATIONS

Comparison of Ion-Pair and Ion-Exchange Liquid Chromatography for Assay of Dalapon Products

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The accuracy of the AOAC official first action ion-pair liquid chromatographic assay for dalapon grasskiller products was studied by comparison with an independent ion-exchange liquid chromatographic method. No statistically significant difference in assay results was seen between the latter method and the AOAC official method for the assay of sodium salt, mixed sodium/magnesium salt, or the free acid forms of dalapon.

The ion-pair liquid chromatographic (LC) method for DOWPON, DOWPON-M,[3] and dalapon acid (2,2-dichloropropionic acid) was successfully collaboratively studied in 1983 (1). DOWPON and DOWPON-M are the sodium and sodium/magnesium salts of dalapon acid, respectively.

In the ion-pair method, the product is dissolved in water. If dilute aqueous mineral acids are used instead of the recommended water, a 2% higher response is noted in samples and standards. The method uses a dalapon acid analytical standard because no high purity standards of DOWPON or DOWPON-M exist. Therefore, the accuracy of the method for DOWPON products was questioned.

We developed an independent ion-exchange LC method and compared it with the ion-pair method for samples of DOWPON, DOWPON-M, and dalapon acid. All work was done using the Hewlett-Packard 1090 LC system with diode array detection as a means of testing this new system for assay work.

Experimental

Ion-Pair Method

The Hewlett-Packard 1090 diode array detector liquid chromatograph system was used, under the following conditions: Eluant: 200 mL acetonitrile, 1.6 mL *n*-octyl amine, 2.4 g $(NH_4)_2HPO_4$ diluted to 1 L with water and adjusted to pH 7.0. Flow rate: 2 mL/min. Column: 4.6 × 250 mm Whatman 10-25-ODS-3 in 40°C column oven. Injection: 20 μL. Detection: 214 nm, 4 nm bandwidth. Integration: HP-3392A, peak height mode. Sample preparation: about 130 mg acid equivalent dalapon acid in 50 mL water (see note below). Standardization: external standardization injection prior to each sample injection. (Note: Standard and samples are hygroscopic; amount of water in dalapon acid standard needs to be determined before use.)

Ion-Exchange Method

The following LC conditions were used: Eluant: 30% v/v aqueous 0.1M H_3PO_4 neutralized to pH 3.0 with 50% NaOH, 30% v/v water, 40% acetonitrile. Flow rate: 2 mL/min. Column: 4.6 × 250 mm DuPont Zorbax amino column in 50°C column oven. Injection: 10 μL. Detector: 214 nm, 4 nm bandwidth. Integrator: HP-3392A, peak height mode. Sample preparation: about 130 mg acid equivalent dalapon acid in 50 mL water (see note below). Standardization: External standardization injection prior to each sample injection. (Note: Standard and samples are hygroscopic and amount of water in dalapon acid standard needs to be determined before use.)

Results and Discussion

Development of Ion-Exchange Method

The obvious alternative check method for the official ion-pair method was one based on ion-exchange analysis. The ion-exchange method of Cortes (2) was adapted for the assay of dalapon products by adding acetonitrile to the eluant and adjusting the pH of the 0.1M phosphate buffer so that all known impurities in dalapon products were resolved from 2,2-dichloropropionic acid. (Note that pyruvic acid eluted at 4.3 min and other peaks present with pyruvic acid were impurities; see Figure 1.)

Selection of check samples.—The following samples were obtained representing the 3 principal product types: DOWPON: Lot 840901; DOWPON-M: Lot 841027; dalapon acid: retainer from tank car Dowx 6100, sampled September 15, 1984.

Precision of analysis.—The HP-1090 instrument was originally used for the ion-exchange LC method by placing 0.1M phosphate buffer in eluant reservoir A, water in reservoir B, and acetonitrile in reservoir C, and programming the instrument to deliver 30% A, 30% B, and 40% C. With this procedure, assay precision for a standard containing 126.8 mg dalapon acid per 50 mL water, injected 16 times, was 2% relative standard deviation (RSD).

An RSD of 2% was judged too high for the purposes of this work. It was suspected that the HP-1090 ternary eluant mixing system delivered eluant with small but nonetheless unacceptable variation in composition. When the eluant was premixed, assay precision for a standard containing 141.7 or 140.6 mg dalapon acid per 50 mL water, each injected 10 times, was 0.25 or 0.37% RSD, respectively. The precision obtained with a premixed eluant was statistically significantly better at the 99% confidence level by the *F*-test. Therefore, all further work was done using premixed eluants.

In a precision study of the AOAC method and premixed eluant, 17 injections of a standard containing 125.5 mg dal-

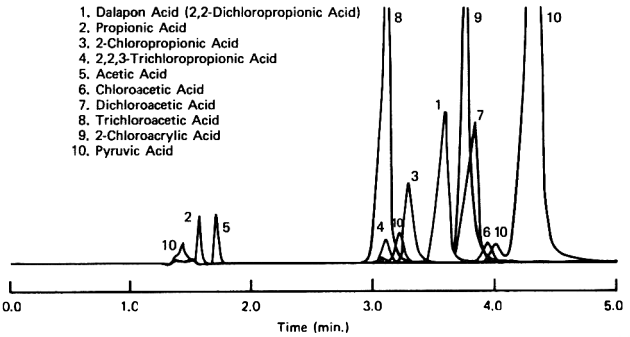

Figure 1. Superimposed chromatograms of dalapon acid and related impurities obtained by ion-exchange method.

Received October 8, 1985. Accepted April 15, 1986.

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[3] DOWPON and DOWPON-M are registered trademarks of the Dow Chemical Co.

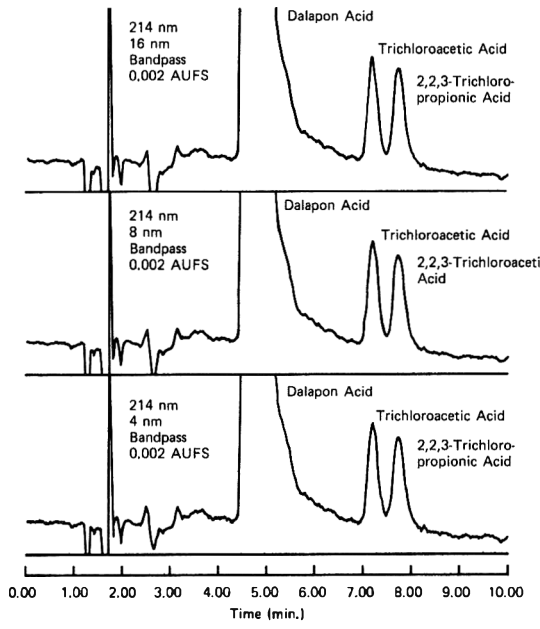

Figure 2. Example chromatograms of single injection of dalapon acid at 0.002 AUFS, 214 nm, and bandwidths of 4, 8, and 16 nm.

apon acid per 50 mL water yielded an RSD of 0.28%. This compares well with the RSD reported for the AOAC collaborative study (1).

Comparative Results

Test samples were assayed in quadruplicate using each method with peak height integration and multipoint calibration (see Table 1). The data indicate no statistically significant difference between the results of the 2 methods at the 95% confidence level.

Sensitivity of Impurity Determination

The HP-1090 system with diode array detection allows the disc storage of up to 8 simultaneous chromatograms from a single injection. Each chromatogram can be recorded at a selected wavelength from 200 to 600 nm with a bandwidth around that wavelength from 4 to 100 nm. For assay work, one generally selects the narrowest bandwidth (4 nm) to maximize linearity. For impurity determinations, a wider bandwidth would be expected to increase the signal-to-noise ratio and thus result in increased sensitivity (3).

Table 1. Assay of dalapon products by ion-pair (AOAC) (1) and ion-exchange (check) methods[a]

Product	Dalapon acid equivalent, %	
	AOAC method	Check method
DOWPON	75.6 ± 0.3	75.8 ± 0.3
DOWPON-M	75.7 ± 0.3	75.9 ± 0.3
Dalapon acid	90.6 ± 0.3	90.6 ± 0.3

[a] Variance reported at 95% confidence level; quadruplicate analyses.

Figure 2 shows 3 chromatograms of a dalapon acid standard, all from a single injection of an extract from the AOAC method, with detection at 214 nm and with bandwidths of 4, 8, and 16 nm. Only a very slight improvement in signal-to-noise ratio was seen at the wider bandwidths. In any event, the baseline noise demonstrated in Figure 2 even at 4 nm bandpass is excellent for a sensitivity of 0.002 AUFS.

Conclusions

No statistically significant difference was seen between the independent check method and the official AOAC method for the assay of DOWPON, DOWPON-M, or dalapon acid. Excellent precision (0.3–0.4% RSD) was obtained with the use of premixed eluant with the HP-1090 system. However, poor precision (2% RSD) was obtained with the use of the 3-reservoir system for a mixed eluant, apparently due to small variations in eluant composition. Excellent signal-to-noise ratios were observed with the HP-1090 diode array detector, at 214 nm, allowing the determination of impurities even at a sensitivity setting of 0.002 AUFS.

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Determination of *N*-Nitrosodimethylamine Levels in Some Canadian 2,4-D Amine Formulations

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Levels of *N*-nitrosodimethylamine (NDMA) were determined in 112 samples of 2,4-dichlorophenoxyacetic acid, (2,4-D), formulated as the dimethylamine salt, collected over a 2 year period from products on the Canadian market. A sample aliquot is partitioned with dichloromethane, and the co-extracted dimethylamine is removed by cleanup on a silica gel column. The eluates containing NDMA are concentrated, an internal standard of *N*-nitrosodipropylamine is added, and nitrosamine levels are determined using a gas chromatograph interfaced with a thermal energy analyzer. Recoveries of NDMA and *N*-nitrosodiethylamine spiked into samples were 103 ± 16 and 96.3 ± 9.8%, respectively. Of the 112 samples analyzed, 92 were below 1 part per million (ppm) relative to the amount of 2,4-D in the samples, 16 were between 1 and 5 ppm, and 4 were greater than 5 ppm. The gas chromatographic column used is compared to a conventional packing material for volatile nitrosamine analysis. Formation of NDMA during cleanup and analysis was shown not to occur.

N-Nitrosamines are known to be carcinogenic to many animal species and have been related to human cancer (1). A number of herbicides used in agriculture, when formulated as the dimethylamine salt, have been shown to contain *N*-nitrosodimethylamine (NDMA) at levels between 0.05 and 640 ppm (2, 3). In Canada, the Pest Control Products Regulations of the Pest Control Products Act set a maximum tolerance of 1 part *N*-nitrosodipropylamine (NDPA) per million parts of trifluralin in formulations (June 1982). Currently, NDMA levels are not regulated in amine salt formulations of phenoxy acid herbicides, such as 2,4-dichlorophenoxyacetic acid (2, 4-D), 2-methyl-4-chlorophenoxyacetic acid (MCPA), and 2-methoxy-3,6-dichlorobenzoic acid (dicamba). This work was undertaken to assess levels of NDMA in 2,4-D products of this type.

Initial laboratory investigations involved setting up a reliable and sensitive method for the determination of NDMA in 2,4-D formulations which was capable of good recoveries and which did not introduce artifacts leading to erroneously high results. The possible contamination or formation of NDMA during sample workup and analysis have been reported previously (4–6), and a suitable method must show that this does not occur. Due to its sensitivity and selectivity toward nitrosamines, a thermal energy analyzer (TEA) interfaced with a gas chromatograph (GC) was used for the detection of NDMA in this study.

METHOD

Materials and Apparatus

(a) *Gas chromatograph.*—Hewlett-Packard 5792 equipped with 7671A autosampler and fitted with 5 ft × 3 mm id glass column packed with 10% Pennwalt 223 + 4% KOH on 80–100 mesh Porapak R (Alltech Associates, Inc., 2051 Waukegan Rd, Deerfield, IL 60015). Operating conditions: injection port 200°C, column oven 160°C, nitrogen carrier flow 30 mL/min.

(b) *Thermal energy analyzer.*—Model 543 with interface temperature 200°C and pyrolyzer temperature 550°C; equipped with CTR gas stream filter (Thermo Electron Corp., 101 First Ave, Waltham, MA 02154).

(c) *Electronic integrator.*—Hewlett-Packard 3390A in peak height mode.

(d) *Chromatographic columns.*—30 cm × 2.3 mm id, fitted with Teflon stopcocks.

(e) *Kuderna-Danish evaporators.*—Kontes Scientific Glassware.

(f) *Silica gel.*—BDH No. M07734, preheated 24 h in oven at 130°C.

(g) *Sodium sulfate, anhydrous.*—Fisher S-421, dried 24 h in oven at 130°C.

(h) *Dichloromethane.*—Distilled in glass (Caledon).

(i) *Ethanol.*—100% (Consolidated Alcohols Ltd).

(j) *Anti-bumping granules.*—BDH No. B33009.

(k) *Nitrosamine standards.*—Working standards prepared from stock 100 μg/mL solutions of individual nitrosamines in acetone, obtained from Thermo Electron Corp.

(l) *Nitrosamine-free deionized water.*—Water passed through Milli-Q system, 18 megaohm-cm (Millipore Corp., 80 Ashby Rd, Bedford, MA 01730). *Caution:* Nitrosamines are carcinogenic. Handle samples and standards in fume hood only. Use of red fluorescent lighting is recommended to avoid possible photodegradation of compounds.

Extraction and Cleanup

Pipet 5 mL aliquot of herbicide amine formulation into 40 mL glass centrifuge tube containing 10 mL nitrosamine-free deionized water. Partition with three 15 mL portions of dichloromethane by shaking 1 min, then centrifuge 5 min to break emulsion, if necessary. Using cleanup column previously packed with 1 cm sodium sulfate, 10 g activated silica gel, and another 1 cm sodium sulfate, remove coextracted dimethylamine by adding dichloromethane extracts, followed by 50 mL 1% absolute ethanol in dichloromethane. Discard these eluates and collect next 75 mL 4% ethanol in dichloromethane directly into Kuderna-Danish apparatus. Add an anti-bumping granule, attach 3-ball Snyder column, and concentrate eluate to 1–5 mL at 50°C. Add known amount of *N*-nitrosodipropylamine (NDPA) as internal standard, mix, and transfer aliquot to autosampler vial for GC analysis.

Recovery checks were done by spiking known amounts of NDMA and *N*-nitrosodiethylamine (NDEA) into a duplicate of every fifth sample, prior to partitioning with dichloromethane. Recovery of NDMA was calculated by subtracting amount of NDMA determined in unspiked sample from total in spiked sample, and comparing this result to amount added.

Table 1. Reproducibility of GC/TEA system with autosampler (*N* = 12)

Nitrosamine	Calibrated amt, pg	Av. reported, pg	SD, %[a]
NDEA	100.0	102.3	1.26
NDPA	100.0	101.8	1.62
NDBA[b]	100.0	101.2	0.975

[a] Standard deviation.
[b] *N*-Nitrosodibutylamine.

Received November 12, 1985. Accepted March 25, 1986.
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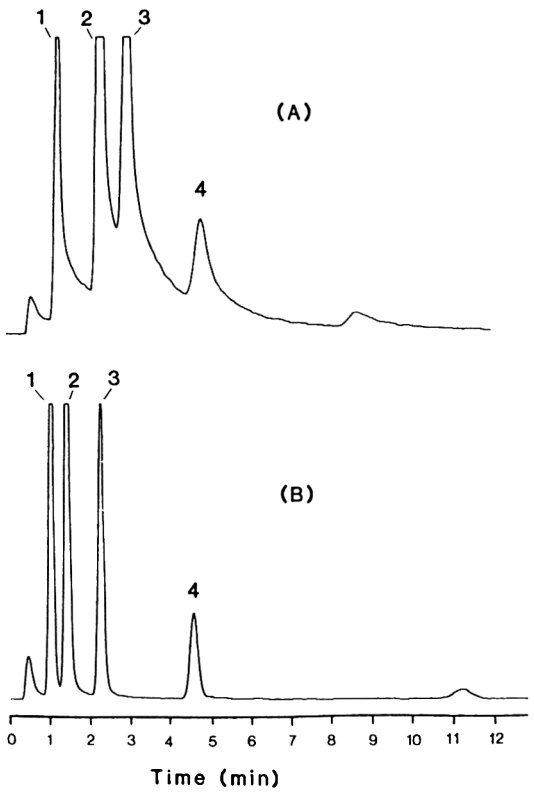

Figure 1. Comparison of (A) 10% Carbowax 20M + 1% NaOH and (B) 10% Pennwalt 223 + 4% KOH for the elution of (1) NDMA, (2) NDEA, (3) NDPA, and (4) NDBA.

No NDEA was observed in any formulations analyzed, so recovered amount of this nitrosamine could be directly measured.

Gas Chromatographic Analysis

Inject 4 μL working standard containing, typically, following concentrations of accurately prepared nitrosamine standards: NDMA and NDEA, 300 ng/mL; NDPA, 500 ng/mL. Calibrate integrator on basis of this injection, repeat the injection, and average the 2 responses if within 2% of each other, otherwise repeat injections. Inject 4 μL of sample aliquots to determine amount of NDMA and NDEA present. Reinject standard to ensure instrument stability.

Results and Discussion

The method described permits the analysis of 5 samples per day, as well as 1 additional formulation spiked with

Table 2. NDMA levels found in 2,4-D formulations: before cleanup, after cleanup,and with sodium nitrite added

	NDMA concn, ppm		
Sample	Before cleanup	After cleanup[a]	Na_2NO_2 added
1	1.52	1.19	1.20
2	1.80	1.53	1.52
3	1.14	0.980	1.00
4	1.17	0.990	1.04
5	3.95	3.54	3.52
6	1.82	1.60	1.51
7	34.7	11.6	11.8
8	0.162	0.142	0.154
9	0.801	0.704	0.723
10	1.71	1.54	1.54
11	0.396	0.332	0.329
12	1.22	1.08	1.10

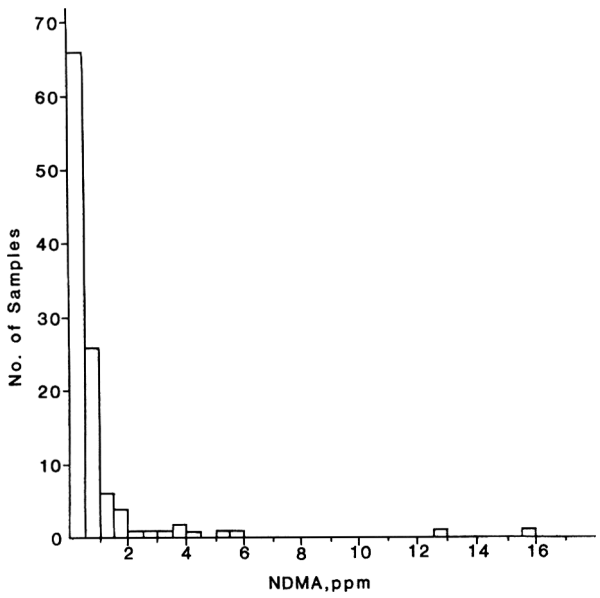

Figure 2. Distribution of NDMA levels in 2,4-D dimethylamine formulations sampled in Canada.

NDMA and NDEA. The use of an autosampler for the GC analysis not only requires less analyst time, it also permits very reproducible quantitation (Table 1). A comparison of stationary phases showed that the 10% Pennwalt 223 + 4% KOH used in this study is superior to 10% Carbowax 20M + 1% NaOH (Figure 1), a widely used phase for this type of analysis. Virtually no tailing is evident with the former packing, with near baseline separation of NDEA and NDPA on a 5 ft column.

Prior to this study, the elution solvent used for silica gel cleanup column consisted of mixed diethyl ether and dichloromethane. At that time, analysis of trifluralin formulations for NDPA contamination was performed in our laboratory according to the method of Maybury and Grant (7). This procedure was modified for the cleanup of 2,4-D formulations, but it was convenient to have the same elution solvents for both determinations. However, a large solvent front was observed during GC analysis when the cleanup columns were eluted with solvent mixtures that had not been freshly prepared. On standing, the interference increased to the extent that tailing of the solvent peak prevented reliable integration of NDMA, although quantitation of the later-eluting NDEA and NDPA was not a problem. Ethanol was substituted for diethyl ether to overcome this interference, and this also eliminated the need to add ethanol as a keeper to prevent losses of volatile nitrosamines during the concentration step.

Fan and Fine (5) have shown that NDMA can be formed in the hot injection port of a gas chromatograph when nitrosamine precursors are present. They also demonstrated that either an 8-fold dilution or an extraction with dichloromethane could eliminate this artifactual formation of NDMA. However, the level of NDMA that they observed in the formulation was 360 ppm, while the samples analyzed in this study range from 0.02 to 15.5 ppm, with a minimum detectable limit of 0.006 ppm. Since dimethylamine is coextracted into dichloromethane during partition, the formation of even a low level of NDMA, resulting from the presence of dimethylamine, would greatly affect the results, and its complete removal was desired. It was also important to show that NDMA was not formed on the silica gel being used to remove the dimethylamine. Table 2 shows the results

before and after silica gel cleanup, using external standards. Using the paired t-test, no significant difference was observed at the 0.05 confidence level, after correction for recoveries of NDEA spiked into the sample extracts prior to cleanup. Sample 7 showed a marked decrease in NDMA concentration following cleanup, which may be due to high levels of available amine in the formulation and its subsequent removal by the silica gel.

To ensure that all of the dimethylamine was removed by the silica gel, a solution of sodium nitrite was prepared by dissolving 1.2 g sodium nitrite in 10 mL nitrosamine-free deionized water. The stock solution was then diluted 1:1000 with ethanol. Following analysis of the herbicide amine extracts by using internal standards, 20 μL of the dilute sodium nitrite was added to the extracts. The results in Table 2 show that there is no residual dimethylamine, which would be apparent by an immediate increase of NDMA following this treatment.

The main objective of this study was to develop a method for analysis of formulations which would accurately reflect the level of NDMA present in the sample, eliminating the possibility of its loss or formation during analysis. We have observed that levels of NDMA increase in some formulations during storage, and studies are in progress to determine the extent to which this occurs and the conditions that influence NDMA formation (8).

Replicate analyses of 2 samples at the 0.8 ppm level gave relative standard deviations of 1.69 ($N = 5$) and 2.71% ($N = 6$), showing that the levels determined are reproducible using this method. Recoveries of spiked NDMA and NDEA throughout this study were 103 ± 16 and 96.3 ± 9.8%, respectively. The higher standard deviation for NDMA recoveries probably results because it is calculated by using the difference between spiked and nonspiked samples run on 2 columns: Slight differences in efficiency between columns can yield significant differences in calculated recovery, especially when the level of NDMA in the original sample is large compared with the spiked amount.

Figure 2 shows the distribution of levels of NDMA found in the 2,4-D dimethylamine formulations. The samples were collected over a 2-year period for other programs in the Food Production and Inspection Branch of Agriculture Canada, and were stored at room temperature. The majority (66) of the 112 formulations analyzed contained no more than 0.5 ppm NDMA relative to the amount of 2,4-D present. An additional 26 contained up to 1.0 ppm, which accounts for 82% of the samples. A rapid falloff in the number of samples is seen as the NDMA concentration increases, with a total of 91% containing less than 2 ppm NDMA.

The results reported in this paper were obtained from samples up to 2 years old. A survey of NDMA levels in dimethylamine formulations of 2,4-D, MCPA, and dicamba currently being sold on the Canadian market is under way, and the results will be reported when it is completed.

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Gas Chromatographic Method for Determination of Cypermethrin in Pesticide Formulations: Collaborative Study

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A gas chromatographic method for determination of total cypermethrin in technical and formulated products has been developed and subjected to a collaborative study involving 15 participating laboratories. Each sample was dissolved in methyl isobutyl ketone containing di(2-ethylhexyl) phthalate as internal standard and analyzed by gas chromatography on a glass column with 3% OV-101 on Chromosorb W-HP. Each collaborator was furnished with reference standard and 4 samples of technical material, 3 emulsifiable concentrates, one wettable powder, and one ultralow volume formulation for analysis. The coefficients of variation of the results obtained ranged from 0.955 to 1.7462%. The method has been adopted as official first action by AOAC.

Submitted for publication July 18, 1986.
This report was presented by the General Referee, J. Launer, at the 99th AOAC Annual International Meeting, Oct. 27–31, 1985, at Washington, DC.
The recommendation of the General Referee was approved by the Committee on Pesticide Formulations and Disinfectants and was adopted by the Association. See the General Referee and Committee reports, *J. Assoc. Off. Anal. Chem.* (1986) **69,** March issue.

Cypermethrin is a mixture of isomers of α cyano-3-phenoxybenzyl (±) *cis,trans*,3-(2,2-dichlorovinyl)-2,2-dimethylcylopropanecarboxylate. The mixed isomers are viscous semisolids, virtually insoluble in water. Formulations occur as emulsifiable concentrates, ULV concentrates, and wettable powders.

The method studied is described in document PY/142mA,

Table 1. Summary of results of collaborative study of GC determination of total cypermethrin content in pesticide formulations[a]

Sample	Overall mean, %	Std dev.	Coeff of var., %	Repeat. (*r*)	Reprod. (*R*)
PY/W Cypermethrin technical	72.852	0.8594	1.1797	1.6987	2.7120
PY/X Cypermethrin technical	93.6345	1.3429	1.4342	2.5989	4.2197
PY/Y Cypermethrin technical	93.3105	1.1451	1.2272	2.2672	3.6138
PY/Z Cypermethrin technical	97.7272	1.7065	1.7462	2.5088	5.1423
PY/AC Cypermethrin emulsifiable concentrate	5.679	0.089	1.550	0.220	0.294
PY/AD Cypermethrin emulsifiable concentrate	22.232	0.212	0.955	0.460	0.683
PY/AE Cypermethrin emulsifiable concentrate	38.249	0.406	1.062	0.586	1.221
PY/AF Cypermethrin wettable powder	12.952	0.082	0.631	0.759	0.759
PY/AG Cypermethrin ultralow volume	5.436	0.069	1.261	0.263	0.269

[a] Mean of results for 15 laboratories. All results were tabulated to 3 significant figures where possible. Results were rejected if one or both of a pair of injections was greater than ±2% of their mean. (All data from one laboratory were rejected.) Day mean and overall mean were calculated with accepted results only. Standard deviation and coefficient of variation were calculated using overall laboratory means. Statistical analyses were carried out following guidelines in ISO 5725. No outliers were found by Dixon's outlier test or by Cochran's maximum variance test. Some data were found to be stragglers by Cochran's test but were included in the statistical analysis. There were no significant differences between day 1 and day 2 results.

PAC/3803m, CIPAC/3171/m, and AOAC **6.B01–6.B05**. The sample is dissolved in methyl isobutyl ketone containing di-(ethylhexyl) phthalate as internal standard.The isomers are separated from other materials by gas chromatography (GC) and detected as one peak by flame ionization. Total content is determined by comparison with calibration solutions.

Collaborative Study

Four samples of cypermethrin technical material, 3 emulsifiable concentrates, one wettable powder, and one ultralow volume formulation were analyzed collaboratively by the proposed GC method.

Cypermethrin in Pesticide Formulations

Gas Chromatographic Method

First Action

CIPAC-AOAC Method

6.B01 ***Principle***

Sample is dissolved in methyl isobutyl ketone contg di-(ethylhexyl) phthalate as internal std; 4 cypermethrin isomers, isolated as one peak, are detd by gas chromatgy with flame ionization detection.

6.B02 ***Apparatus and Reagents***

(a) *Gas chromatograph with recorder and integrator.*—With flame ionization detector and 1.0 m × 4 mm (id) glass column packed with 3% OV-101 on 100–120 or 80–100 mesh Chromosorb WHP, capable of on-column injection. Condition newly packed column overnight at 260° with low N flow. Operating conditions: temps—inlet 250°, column 230–240°, detector 250°; carrier gas flow to elute internal std at ca 5.5 min and cypermethrin at ca 11.5 min with ≥30 mm between intercepts of tangents on baseline of std and internal std peaks; adjust H and air for detector as recommended by manuf.; adjust sensitivity to give peak hts 75% full scale.

(b) *Di-(2-ethylhexyl) phthalate (DEHP; dioctyl phthalate).*—Fisher Scientific Reagent, or equiv.

(c) *Methyl isobutyl ketone (MIBK).*—GC quality (J.T. Baker Chemical Co. Reagent, or equiv.).

6.B03 ***Preparation of Standards***

(a) *Internal std soln.*—20 mg DEHP/mL. Weigh ca 10 g DEHP into 500 mL vol. flask, dil. to vol. with MIBK, and mix (soln I). Concn may be varied to accommodate column and instrument differences. If necessary, adjust concn so that peak ht or area of DEHP closely matches peak ht or area of cypermethrin within 10%.

(b) *Cypermethrin std soln.*—4.0 mg/mL. Warm sealed bottle of cypermethrin std (ICI-Americas, Inc.) at 40–50° until no crystals remain; shake bottle. Accurately weigh, in duplicate, ca 0.2 g std into 50 mL vol. flask, and dissolve in 3–4 mL MIBK. Pipet 10.0 mL internal std soln into each flask, dil. to vol. with MIBK, and mix (solns CA, CB). Similarly, weigh ca 0.1 g cypermethrin std into 25 mL vol. flask, dil. to vol. with MIBK, and mix (soln CO).

6.B04 ***Preparation of Sample***

(a) *Technical formulations.*—Proceed as above under cypermethrin std soln, using sample wt contg ca 0.2 g cypermethrin (solns SA, SB, SO).

(b) *Wettable powders.*—Accurately weigh, in duplicate, sample contg 0.2 g cypermethrin into 50 mL vol. flask, pipet in 10.0 mL internal std soln, and add sufficient MIBK to suspend powder. Thoroly shake flask 10 min, dil. to vol. with MIBK, and let powder settle or centrf. until clear. Similarly, prep. soln without internal std, using sample contg ca 0.1 g cypermethrin/25 mL MIBK.

(c) *Ultra-low volume formulations.*—Proceed as above under technical formulations, beginning "Accurately weigh . . .".

(d) *Emulsifiable concentrates.*—Proceed as above under wettable powders.

6.B05 ***System Performance Check and Determination***

Using instrument conditions listed under *Apparatus and Reagents*, inject 1.5 μL portions of solns I, CO, and SO onto column and check for interfering peaks. On-column injection is necessary. Inject std soln CA and adjust parameters to give peak ht ca 75% full scale with peak quality and elution time specified.

Inject 1.5 μL portions of std solns CA and CB until response ratio (area cypermethrin peak/area internal std peak) varies <0.5% of mean. (Area measurements by digital electronic integration are preferred over other methods.) Carry out injections of std and sample solns in following sequence: CA_1, SA_1, SA_2, CB_1, CA_2, SB_1, SB_2, CB_2. Average response ratios for sample and stds that bracket each sample. Successive response ratios should agree ±5% of their mean. If not, repeat analysis.

$$\text{Cypermethrin, \%} = (R/R') \times (W'/W) \times P$$

where R and R' = av. peak area ratios for sample and std, resp.; W' = g cypermethrin in std soln; W = g sample extd for analysis; and P = % purity of std.

CAS-52315-07-8 (cypermethrin)

Results and Recommendation

The results of the collaborative study are summarized here.

Material	*Nominal concn range, % m/m*	*Repeatability (r)*	*Reproducibility (R)*
TC	98–90	2.5	4.3
TC	72	1.7	2.7
EC	40–20	0.5	1.0
EC	5	0.2	0.3
WP	13	0.8	0.8
UL	5	0.26	0.27

More detailed summary results are given in Table 1. The Panel considered the results to be satisfactory and recommended acceptance as a CIPAC method. The General Referee recommends adoption as an AOAC official first action method.

Acknowledgments

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Gas Chromatographic Method for Determination of Permethrin in Pesticide Formulations: Collaborative Study

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Collaborators: M. Akerblom; R. J. Anderson; P. G. Baker; T. J. Beckmann; A. L. Bertrand; P. D. Bland; R. C. Bruce; B. Crozier; C. Dickinson; W. Dobrat; K. Eden; D. S. Farrington; P. Hitos; N. Janes; G. Livanos; R. J. Parker; K. Pavel; H. H. Povlsen; F. Sanchez-Rasero; C. Self; D. B. Sergeant; G. R. Raw, *Secretary*

A gas chromatographic method for determination of permethrin in technical and formulated products has been developed and subjected to a collaborative study involving 19 participating laboratories. Each sample was dissolved in methyl isobutyl ketone containing *n*-octacosane as internal standard and analyzed by gas chromatography on a glass column with 3% OV-210 on Chromosorb W-HP. Each collaborator was furnished with reference standard and 5 samples of technical material (90-95%), 8 emulsifiable concentrates (10-50%), 2 wettable powders (20-30%), one dustable powder (1-2%), and one water-dispersible granules (1-2%) for analysis. The coefficients of variation of the results obtained ranged from 0.79 to 4.24%. The method has been adopted as official first action by AOAC.

Permethrin is a mixture of the 2 diastereoisomeric forms of 3-phenoxybenzyl 3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate, each of which is present as a pair of enantiomers. The ratio of the 2 enantiomers in each diastereoisomer is 1:1; the ratio of *cis* to *trans* stereoisomers should be declared on the formulation. The technical material is a yellow-brown liquid which partially crystallizes at ambient temperature. Formulations occur as emulsifiable concentrates, solutions for fogging, ULV concentrates, dustable powders, wettable powders, water-dispersible granules, and mosquito coils.

The method studied is described in document PY/129m, PAC/3750m, CIPAC/3169/m, and AOAC **6.B06-6.B11**. The sample is dissolved in 4-methylpentan-2-one containing *n*-octacosane as internal standard. The *cis* and *trans* isomers are separated by gas chromatography and detected by flame ionization. Total content and *cis* and *trans* ratios are determined by comparison with calibration solutions.

Collaborative Study

Five samples of permethrin technical material, 8 emulsifiable concentrates, 2 wettable powders, one dustable powder, and one water-dispersible granules were analyzed collaboratively by the proposed GC method.

Submitted for publication July 18, 1986.

This report was presented by the General Referee, J. Launer, at the 99th AOAC Annual International Meeting, Oct. 27-31, 1985, at Washington, DC.

The recommendation of the General Referee was approved by the Committee on Pesticide Formulations and Disinfectants and was adopted by the Association. See the General Referee and Committee reports, *J. Assoc. Off. Anal. Chem.* (1986) **69**, March issue.

Table 1. Summary of results of collaborative study of GC determination of total permethrin content in pesticide formulations[a]

Sample	Mean		Overall mean	Std dev.	Coeff. of var., %	Repeat. (*r*)	Reprod. (*R*)
	Day 1	Day 2					
PY/A Permethrin technical	91.46	91.43	91.44	1.225	1.340	1.865	3.709
PY/B Permethrin technical	90.71	90.66	90.68	1.142	1.260	2.836	3.805
PY/C Permethrin technical	93.24	93.03	93.14	1.305	1.401	3.768	4.554
PY/D Permethrin technical	95.17	95.12	95.14	0.953	1.002	2.202	3.114
PY/E Permethrin technical	91.78	91.95	91.87	1.154	1.256	1.979	3.553
PY/J Permethrin EC	47.68	47.61	47.65	0.377	0.791	1.681	1.681
PY/K Permethrin EC	38.62	38.70	38.66	0.347	0.898	1.480	1.480
PY/L Permethrin EC	20.16	20.20	20.18	0.315	1.562	0.617	0.993
PY/M Permethrin EC	22.36	22.54	22.45	0.226	1.005	1.182	1.182
PY/N Permethrin EC	11.33	11.43	11.38	0.482	4.239	0.622	1.434
PY/O Permethrin EC	26.73	26.75	26.74	0.634	2.370	1.067	1.946
PY/P Permethrin EC	21.89	22.02	21.95	0.478	2.176	1.038	1.538
PY/Q Permethrin EC	10.93	10.94	10.93	0.189	1.725	0.600	0.682
PY/R Permethrin WP	25.26	25.46	25.36	0.250	0.985	0.760	0.888
PY/S Permethrin WP	21.24	21.48	21.36	0.237	1.110	1.294	1.294
PY/T Permethrin DP	1.009	1.019	1.014	0.018	1.743	0.054	0.063
PY/U Permethrin WG	1.443	1.459	1.451	0.053	3.633	0.178	0.195

[a] Mean of results for 19 laboratories. All results were tabulated to 3 significant figures where possible. Results were rejected if one or both of a pair of injections was greater than ±2% of their mean. Day mean and overall mean were calculated with accepted results only. Standard deviation and coefficient of variation were calculated using overall laboratory means. Statistical analyses were carried out following guidelines in ISO 5725. There were no significant differences between day 1 and day 2 results.

Permethrin in Pesticide Formulations

Gas Chromatographic Method

First Action

CIPAC-AOAC Method

6.B06 *Principle*

Sample is dissolved in methyl isobutyl ketone contg *n*-octacosane as internal std, and permethrin is detd as total area of 2 isomer peaks by gas chromatgy with flame ionization detection.

6.B07 *Apparatus and Reagents*

(a) *Gas chromatograph with recorder and integrator.*—With flame ionization detector and 1.0 m × 4 mm (id) glass column packed with 3% OV-210 on 100–120 or 80–100 mesh Chromosorb WHP, capable of on-column injection. Condition newly packed column overnight at 275° with low N flow. Operating conditions: temps—inlet 260°, column 190–220°, detector 250°; carrier gas flow to elute internal std in ca 4.0 min and *trans*-permethrin at ca 9.5 min with ≥30 mm between intercepts of tangents on baseline of internal std and std *cis*- and *trans*-isomer peaks; adjust H and air for detector as recommended by manuf.; adjust sensitivity to give peak hts 75% full scale.

(b) *n-Octacosane std.*—With no peaks at retention times of permethrin isomers (Kodak Laboratory Chemicals, or equiv.).

(c) *Methyl isobutyl ketone (MIBK).*—GC quality (J.T. Baker Chemical Co. Reagent, or equiv.).

6.B08 *Preparation of Standards*

(a) *Internal std soln.*—1 mg *n*-octacosane/mL. Weigh ca 0.5 g *n*-octacosane into 500 mL vol. flask, dissolve in 300 mL MIBK, dil. to vol. with MIBK, and mix (soln I). Concn may be varied to accommodate column and instrument differences. If necessary, adjust concn so that peak ht of *n*-octacosane closely matches peak ht of permethrin isomers.

(b) *Permethrin std soln.*—4.0 mg/mL. Warm sealed bottle of permethrin std (ICI-Americas, Inc.) at 40–50° until no crystals remain; shake bottle. Accurately weigh, in duplicate, ca 0.1 g std into 100 mL g-s erlenmeyer. Pipet 25.0 mL internal std soln into each flask and shake until permethrin is dissolved (solns CA, CB). Similarly, weigh ca 0.1 g permethrin std into 25 mL vol. flask, dissolve in 15 mL MIBK, dil. to vol. with MIBK, and mix (soln CO).

6.B09 *Preparation of Sample*

(a) *Technical formulations.*—Proceed as above under permethrin std soln, using sample wt contg ca 0.1 g permethrin (solns SA, SB, SO).

(b) *Wettable and dustable powders (suspendibility >50%).*—Accurately weigh, in duplicate, sample contg 0.1 g permethrin into 100 mL g-s erlenmeyer. Pipet 25.0 mL internal std soln into flask, stopper, and shake thoroly 10 min. Let settle, filter thru Whatman No. 54 paper into g-s flask, and use filtrate for analysis. Similarly, prep. soln without internal std, using sample contg ca 0.1 g permethrin/25 mL MIBK.

(c) *Emulsifiable concentrates.*—Proceed as above under wettable and dustable powders.

(d) *Water-dispersible granules.*—Grind ca 20 g sample to fine powder and thoroly mix. Accurately weigh, in duplicate, sample contg 0.1 g permethrin into 100 mL g-s erlenmeyer. Pipet 25 mL internal std soln into flask and place in ultrasonic bath 10 min. Proceed as above under wettable and dustable powders, beginning "Let settle . . . ".

6.B10 *System Performance Check*

Using instrument conditions listed under *Apparatus and Reagents*, inject 3 or more 1.5 μL portions of soln CA onto column and adjust parameters to give peak ht ca 75% full scale with peak quality and elution time specified. On-column injection is necessary. Inject 1.5 μL solns I, CO, and SO and check for interfering peaks.

6.B11 *Determination*

Inject 1.5 μL std solns CA and CB until response ratio (total area of *cis*- and *trans*-permethrin peaks/area internal std peak) varies <0.5% of mean. (Area measurements by digital electronic integration are preferred over other methods.) Carry out injection of std and sample solns in following sequence: CA_1, SA_1, SA_2, CB_1, CA_2, SB_1, SB_2, CB_2. Average response ratios for sample and stds that bracket each sample. Successive response ratios should agree ±5% of their mean. If not, repeat analysis.

$$\text{Permethrin, \%} = (R/R') \times (W'/W) \times P$$

where R and R' = av. peak area ratios for sample and std, resp.; W' = g permethrin in std soln; W = g sample extd for analysis; and P = % purity of std.

CAS-52645-53-1 (permethrin)

Results and Recommendation

The results of the collaborative study are summarized here.

Material	Nominal concn range, % m/m	Repeatability (r)	Reproducibility (R)
TC	95–90	2.7	3.6
EC	50–26	1.4	1.7
EC	25–10	0.8	1.2
WP	30–20	1.0	1.1
DP	2–1	0.05	0.06
WG	2–1	0.18	0.20

More detailed summary results are given in Table 1. The Panel considered the results to be satisfactory and recommended acceptance as a CIPAC method. The General Referee recommends adoption as an AOAC official first action method.

Acknowledgments

The following members of the panel were associated with the collaborative work:

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Gas Chromatographic Determination of Isofenphos in Technical and Formulated Products: Collaborative Study

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A gas chromatographic method for the determination of isofenphos (OFTANOL®) in isofenphos technical and liquid formulations has been developed and collaboratively studied by 11 laboratories. Two technical samples and 2 liquid flowable samples were analyzed after shaking/extracting in methanol which contained diisobutyl phthalate as an internal standard. The extracts were analyzed by gas chromatography using an SP-2100 column and either flame ionization or thermal conductivity detection. Coefficients of variation were 0.57 and 1.27% for the technical and formulated products, respectively. The GC method has been adopted official first action as a AOAC-CIPAC method.

Isofenphos (OFTANOL®), 1-methylethyl 2-[[ethoxy[(1-methylethyl)amino]phosphinothioyl]oxy]benzoate, is available as a 5% active ingredient granule (5G) and a 2 lb/gal. active ingredient flowable (2F) formulation. Isofenphos is an organophosphorus pesticide which is highly effective for control of soil-inhabiting insects. Although a few residue and metabolite methods have been reported for isofenphos, no quantitative chromatographic method has been published for isofenphos active ingredient in technical or formulated products.

A gas chromatographic (GC) method using diisobutyl phthalate (DIBP) as an internal standard was developed using either thermal conductivity (TCD) or flame ionization detection (FID). This paper describes a collaborative study on the method to evaluate its precision, reproducibility, and functionality.

Collaborative Study

Eleven laboratories agreed to participate in the study. Two different samples each of isofenphos technical and 2F formulation, analytical standard, internal standard, and SP-2100 column packing (if needed) were sent to each laboratory. The

Submitted for publication June 5, 1986.

This report of the Associate Referee was presented at the 99th AOAC Annual International Meeting, Oct. 27–31, 1985, Washington, DC.

The recommendation of the Associate Referee was approved by the General Referee and the Committee on Pesticide Formulations and Disinfectants and was adopted by the Association. See the General Referee and Committee reports, *J. Assoc. Off. Anal. Chem.* (1987) **70**, March issue.

OFTANOL is a registered trademark of the parent company of Farbenfabriken Bayer GmbH, Leverkusen, FRG.

Table 1. Collaborative results for GC analysis of isofenphos 2F formulation[a]

Coll.	Isofenphos, % A	Isofenphos, % B	Diff. (D) (A − B)	Total (T)
1	23.30	23.00	0.30	46.30
2	23.31	23.54	−0.23	46.85
3	23.34	23.07	0.27	46.41
4	23.30	23.34	−0.04	46.64
5	23.53	23.09	0.44	46.62
6	23.30	23.20	0.10	46.50
7	23.38	23.18	0.20	46.56
8	23.20	22.38	0.82	45.58
9	23.08	23.74	−0.66	46.82
10	23.36	23.80	−0.44	47.16
11	23.38	24.20[b]		
Mean	23.32	23.23		
Std dev.	0.11	0.39		
S_r				0.30
S_o			0.30	
S_L				0.00
CV_r, %				1.27

[a] Closely matched pairs.

[b] This value is not included in the statistical evaluation due to unusual appearance of sample reported by collaborator.

Table 2. Collaborative results for GC analysis of isofenphos technical product[a]

Coll.	Isofenphos, % A	Isofenphos, % B	Diff. (D) (A − B)	Total (T)
1	91.91	91.46	0.45	183.37
2	91.38	91.48	−0.10	182.86
3	91.96	91.61	0.35	183.57
4	92.02	91.54	0.48	183.56
5	90.76	91.72	−0.96	182.48
6	92.00	91.86	0.14	183.86
7	91.62	91.78	−0.16	183.40
8	92.56	92.36	0.20	184.92
9	93.22	91.72	1.50	184.94
10	92.16	91.62	0.54	183.78
11	92.08	91.86	0.22	183.94
Mean	91.97	91.73		
Std dev.	0.62	0.25		
S_r				0.48
S_o			0.43	
S_L				0.21
CV_r, %				0.53

[a] Closely matched pairs.

collaborative study design was carried out according to suggestions of Youden and Steiner (1).

Isofenphos Technical and in Pesticide Formulations

Gas Chromatographic Method

First Action

AOAC-CIPAC Method

(Method is suitable for tech. isofenphos and formulations with isofenphos as only active ingredient.)

Principle

Isofenphos is extd with MeOH contg diisobutyl phthalate as internal std, analyzed by gas chromatgy with flame ionization or thermal conductivity detection, and quantitated by comparing peak areas (or hts) of sample and internal std.

Apparatus and Reagents

(a) *Gas chromatograph.*—Equipped with thermal conductivity (TC) detector or flame ionization detector (FID). *Operating conditions:* Temps—inlet 250°, column 190°, detector 250°; carrier gas 20–30 mL/min (He for TC detector, either He or N for FID); bridge current 180 mA or as recommended for TC detector; air and H flows as recommended for FID; chart speed 1.0 cm/min; range 1 (10 for FID); attenuation (×2) (×8 for FID). Retention times: internal std ca 1.7 min, isofenphos ca 3.5 min. Let chromatograph stabilize (flat baseline) before beginning injections and allow ca 5 min run time for each injection.

(b) *Chromatographic column.*—0.5 m × 2 mm id stainless steel or glass column packed with 10% SP-2100 on 80–100 mesh Supelcoport or equiv. support.

(c) *Diisobutyl phthalate.*—Kodak Laboratory Chemicals (Eastman Kodak) No. 6830 or equiv. that contains no impurities eluting at retention time of isofenphos.

(d) *Isofenphos reference std.*—Mobay Corp.

(e) *Internal std soln.*—Pipet 10 mL diisobutyl phthalate into 1 L vol. flask, dil. with MeOH, and mix well. If necessary, adjust concn so that peak hts of isofenphos and internal std are within 20% (±10%).

(f) *Filters.*—0.45 μm porosity (Gelman Acrodisc-CR, or equiv.).

Preparation of Standard

Accurately weigh ca 250 mg ref. std isofenphos into glass bottle (ca 50 mL for TC detector or 150 mL for FID). Pipet in 15 mL internal std soln. For FID, add addnl 100 mL MeOH. Cap securely and mix well.

Preparation of Sample

Accurately weigh sample contg ca 250 mg isofenphos into glass bottle (ca 50 mL for TC detector or 150 mL for FID). Pipet in 15 mL internal std soln. For FID, add addnl 100 mL methanol. Cap securely and mix well to ext.

Adjust operating parameters so that isofenphos elutes in 3.2–3.7 min. Adjust injection vol. and attenuation to give largest on-scale peaks. Make successive 2 μL injections of std soln until response is stable and response ratios (R) of isofenphos peak area (ht) to internal std peak area (ht) agree within ±1% of their mean.

Make duplicate 2 μL injections of each sample. Ratios of isofenphos to internal std peak area (ht) must agree within ±1% of their mean. If not, repeat detn, starting with std injections.

After every 4–6 sample injections, and/or after last sample injection, make 2 successive injections of ref. std soln to bracket samples. Av. std ratios preceding and following bracketed samples must lie within ±1% of mean; otherwise, repeat series of injections.

Calculation

$$\text{Isofenphos, wt \%} = (R/R') \times (W'/W) \times P$$

where R and R' = av. response ratios for sample and ref. std solns, resp.; W' and W = wt (mg) of isofenphos std and sample solns, resp.; and P = purity of isofenphos std (%).

CAS-25311-71-1 (isofenphos)

Results and Discussion

Each of the 11 collaborating laboratories submitted a complete set of results. These are shown in Tables 1 and 2.

A variety of equipment was used for the analyses including at least 4 different brands of gas chromatographs. Two participants used TC detection; the others used flame ionization detection. Both glass and metal columns were used in lengths varying from 12 to 24 in. One collaborator successfully used a different column packing (SE-30); all others used SP-2100 packing. Two collaborators used peak height measurements for quantitation; the others used peak area.

Isofenphos is a moderately polar, high-boiling compound. Therefore, short nonpolar (methyl silicone) columns are required to resolve isofenphos from its impurities and degradation products. The analysis is performed in methanol because of the solubility of the 2F formulation (aqueous). The internal standard, diisobutyl phthalate, contained a minor impurity which eluted closely behind the DIBP peak. Two

collaborators preferred peak height measurements because of integration difficulties with the impurity peak; however, the remaining collaborators experienced no difficulty with peak areas. (Two collaborators who performed peak height *and* peak area measurements found insignificant differences.) Incidentally, the wide variety of data systems used by different laboratories presents a problem in describing uniform peak area measurement. Analysts are advised to optimize the appropriate integration events of their data system to integrate the peak data reproducibly, and when in doubt to use peak heights.

Results agreed well among the laboratories. The average standard deviation (S_x) for the technical material was 0.48 and 0.30 for the formulation. The reproducibility coefficient of variation (CV_x) was 0.53% for the technical material and 1.27% for the formulation. Collaborator 11 reported residue and gelatinous material inside the cap of that sample container, but did not notice it until after the analysis was completed. Those data were not included in the statistical evaluation. No values were rejected by either the ranking test or the Dixon test (2).

Recommendation

It is recommended that the GC method for determination of isofenphos technical and formulated products be adopted official first action as a AOAC-CIPAC method.

Acknowledgments

The author thanks the following collaborators and their associates for their cooperation in this study:

M. Bender, Bayer AG, Leverkusen, FRG

Oliver O. Bennett, Jr, Kansas Dept of Agriculture, Topeka, KS

David Carlson, McLaughlin Gormley King Co., Minneapolis, MN

A. A. Carlstrom and John R. Updyke, Chevron Chemical Corp., Richmond, CA

James J. Karr and Eugene DiPilla, Pennwalt Corp., King of Prussia, PA

D. H. Koenig, Lilly Research Laboratory, Greenfield, IN

Paul Korger and Talora Zank, Wisconsin Dept of Agriculture, Madison, WI

Thomas H. Riggs, Mobay Corp., Kansas City, MO

Michael Sabbann, Minnesota Dept of Agriculture, St. Paul, MN

Ray Schulz, Indiana State Laboratory, West Lafayette, IN

Laszlo Torma and Heidi Hicks, Montana Dept of Agriculture, Bozeman, MT

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ARTIFICIAL SWEETENERS

Liquid Chromatographic Determination of Saccharin in Beverages and Sweets: NMKL[1] Collaborative Study

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Collaborators: K. Berg-Nielsen; K. Blomberg; M. Guldborg; T. Hagebø; R. Lundqvist; P. Mattsson; E.-L. Niemi; T. Savioja

A reverse phase liquid chromatographic method for the determination of saccharin in a soft drink and a juice was collaboratively studied in 8 laboratories. Collaborators were supplied with 3 samples of the soft drink and 3 samples of the juice containing sodium saccharin levels of 40–100 mg/L. Average recoveries of sodium saccharin were 95.3% for the soft drink and 98.0% for the juice. The reproducibility coefficients of variation were 16.9% for the soft drink and 10.4% for the juice. In addition, a mini-collaborative study was conducted for the determination of saccharin in 3 samples of sweets produced commercially. Five collaborators analyzed the samples, which contained saccharin at levels of 100–600 mg/kg according to the maker's specifications. Saccharin was extracted with water and ethanol and chromatographed using a modified liquid chromatographic method. The reproducibility coefficient of variation was 12.4% for the sweets.

Saccharin is used as an artificial sweetener in soft drinks, juices, sweets, jams, and desserts. The isolation of an acidic compound such as saccharin from foods may be time-consuming. In the official AOAC method, saccharin, sodium benzoate, and caffeine are determined simultaneously by direct injection of an aqueous sample and liquid chromatography (LC) (1). For aqueous samples such as drinks this approach is very suitable. For samples such as sweets and desserts containing food thickeners another method is needed. For example, one method for determining saccharin in foods such as desserts uses differential pulse polarography after isolation of saccharin with Celite (2).

In the present method, saccharin is extracted and food thickeners such as polysaccharides are precipitated with ethanol. A modified LC technique, incorporating step-gradient elution, is used to complete the separation of saccharin from the preservatives. The applicability of the method was tested by spiking samples of sweets. In addition, the capability of the method to detect saccharin in samples containing polysaccharides was tested in a series of 91 desserts served in hospitals. These were of 3 types: berry- or fruit-based or materials such as milk and chocolate, and contained saccharin at levels of 0–220 mg/kg.

Collaborative Study

This paper describes a collaborative study with soft drink and juice samples and a mini-collaborative study with sweets. Eight collaborators analyzed the drinks and 5 analyzed the sweets. The number of participants in the mini-collaborative study was small because only a few laboratories in the Nordic countries were using a modified LC technique routinely.

Three samples of soft drink and 3 samples of juice were spiked with sodium saccharin at levels ranging from 40 to 100 mg/L (see Table 1). The soft drink was a commercial grapefruit drink, consisting of the following components: water, sugar, carbonic acid, and natural aroma of grapefruit. The additives were sodium benzoate (160 mg/kg), citric acid, food color, and sucrose diacetate hexaisobutyrate. The juice was a commercial orange juice consisting of orange juice and sugar. The additives were citric acid, food thickeners, potassium sorbate, and sodium benzoate (both of the latter at 90 mg/kg).

Three samples of sweets were obtained from commercial manufacturers. The main components of the sweets were gum arabic and sorbitol (about 90%). The exact concentrations of saccharin were unknown; the declared levels were

Table 1. Results (mg/L) of LC determination of sodium saccharin

	Juice			Soft drink		
Coll.	Level 1	Level 2	Level 3	Level 1	Level 2	Level 3
1	43	79	83	66	106	78
	43	78	82	69	107	77
	42	78	83	66	107	78
2	38	73	78	48	95	65
	39	73	77	48	96	66
	38	73	78	47	94	65
3[a]	23	47	48	31	46	42
	21	43	47	33	50	36
	21	53	49	33	51	40
4	40	79	159[b]	42	94	53
	37	75	154[b]	43	91	52
	42	75	156[b]	41	83	52
5	40	72	77	42	87	57
	39	72	77	43	87	57
	40	75	78	45	88	58
6	61[b]	93	93	48	91	62
	59[b]	92	92	47	93	61
	60[b]	92	90	47	93	61
7	42	82	86	58	117	74
	42	80	87	61	116	79
	42	81	87	58	116	78
8	37	71	76	36	70	49
	36	73	78	36	75	49
	39	70	79	37	73	49
Added, mg/L	40.0	80.0	85.0	50.0	100.0	67.0
Av. rec., mg/L	39.0	77.9	82.3	48.9	94.2	62.9
Av. rec., %	99.8	97.4	96.8	97.8	94.2	93.9
Std dev.						
Between labs	1.9	7.2	5.9	10.5	13.9	11.2
Within labs	1.3	1.3	1.0	1.2	2.4	1.1
Overall	2.3	7.3	6.0	10.6	14.2	11.3
Reproducibility CV, %	5.8	9.4	7.3	21.7	15.1	17.9
Repeatability CV, %	3.2	1.7	1.2	2.4	2.6	1.8

[a] All results excluded from calculation of precision parameters by Youden's ranking test.
[b] Results excluded by Dixon's test for outliers.

Received December 3, 1985. Accepted April 2, 1986.
This method was accepted as an official NMKL method at the 38th Annual Meeting of the Nordic Committee on Food Analysis (NMKL), 1984.

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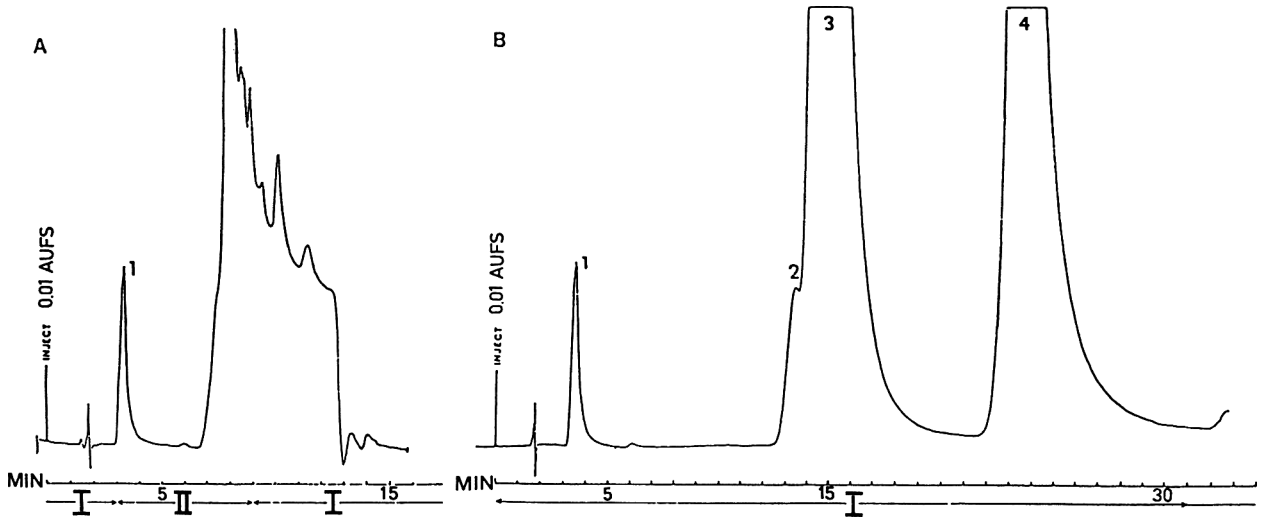

Figure 1. LC chromatograms of 50 mg/L of sodium saccharin (1), and 100 mg/L each of benzoic acid (2), sorbic acid (3), and methyl ester of *p*-hydroxybenzoic acid (4). Mobile phases in A and B: I: acetic acid (1%)-methanol (95 + 5); II: methanol-acetic acid (1%) (70 + 30).

100, 300, and 600 mg/kg. Before homogenization, the sweets were first diluted with water (1 + 10).

Collaborators also received a sodium saccharin standard. They were requested to analyze the samples in triplicate. The soft drink and juice samples were to be analyzed by using one mobile solvent and the sweets by using 2 mobile solvents. Participation in the latter part of the collaborative study was optional.

METHOD

Principle

Saccharin is determined in soft drinks and juices by reverse phase LC with UV detection at 254 nm; sweets must first be extracted with ethanol.

Table 2. Results (mg/kg) of LC determination of sodium saccharin

Coll.	Sweets		
	Level 1	Level 2	Level 3
1	273	496	103
	268	530	103
	279	539	103
2	271	428	79
	268	458	76
	265	428	77
3[a]	54	82	23
	48	79	20
	46	88	23
5	242	484	70
	253	484	70
	253	484	72
7	275	470	152
	275	537	162
	284	—	165
Declared, mg/kg	336	672	112
Av. results, mg/kg	267.1	485.3	102.7
Av. rec. (decl.), %	79	72	92
Std dev.			
Between labs	12.2	34.4	40.4
Within labs	5.2	23.5	3.5
Overall	13.3	41.7	40.6
Reproducibility CV, %	5.0	8.6	39.5
Repeatability CV, %	1.9	4.8	3.4

[a] All results excluded by Dixon's test for outliers.

Apparatus

(a) *Liquid chromatograph.*—Isocratic instrument with possibility of using 2 mobile phases, step-gradient, or gradient system (e.g., Waters Associates, Inc.). Operating conditions: column temperature ambient; flow rate 2.0 mL/min; wavelength 254, 280, 207, or 214 nm; injection volume 10–30 μL; sensitivity 0.005–0.02 AUFS; chart speed 1 cm/min.

(b) *Reverse phase LC column.*—Particle size 10 μm, 30 cm × 3.9 mm id, e.g., μBondapak C_{18} (Waters Associates, Inc.).

(c) *Guard column.*—Particle size 37–50 μm C_{18} (e.g., Waters Associates, Inc.).

(d) *Membrane filters.*—For aqueous solutions, pore size 0.45 μm.

(e) *Centrifuge.*—Minimum 2000 *g*.

Reagents

(a) *Mobile phases.*—Use LC grade methanol and water. Prepare 1% (v/v) acetic acid solution. Mobile phase I: Mix 950 mL 1% acetic acid solution and 50 mL methanol; de-gas. Mobile phase II: Mix 300 mL 1% acetic acid solution and 700 mL methanol; de-gas.

(b) *Ethanol.*—99%.

(c) *Sodium saccharin standard solution.*—100 mg/L. Transfer exactly 25 mg sodium saccharin dihydrate, $C_7H_4NNaO_3S \cdot 2H_2O$ (e.g., BDH Laboratory Reagent), to 250 mL volumetric flask and dilute to volume with water.

Sample Preparation

(a) *Soft drinks and juices.*—Decarbonate, filter if particulate matter is present, and inject.

(b) *Sweets.*—Weigh 10.00 g sample containing ≥0.5 mg

Table 3. Statistical results for LC determination of sodium saccharin in juice and soft drink (mg/L) and in sweets (mg/kg), pooled precision parameters

Statistic	Juice	Soft drink	Sweets
Std dev.			
Between labs	6.5	10.8	20.7
Within labs	1.2	1.7	13.5
Lab.-level interaction	2.0	3.7	24.2
Overall	6.9	11.6	34.6
Reproducibility CV, %	10.4	16.9	12.4
Repeatability CV, %	1.8	2.6	4.8

saccharin and add 100.0 g water. Dissolve sample with mixing. Weigh 10.00 g homogenate into centrifuge tube and add 50 mL ethanol. Mix and let stand overnight. Centrifuge and decant aqueous ethanol phase. Wash precipitate twice with 30 mL ethanol and combine washings with aqueous ethanol phase. Evaporate to dryness and dissolve and dilute residue to 5.00 mL with water. Filter and inject solution directly.

Determination

Inject 10 μL standard solution to determine peak height of saccharin. Repeat injections until results agree within 2%. Inject sample solution containing ca 1 μg saccharin twice. Chromatograph samples from sample preparation (a) with mobile phase I (Figure 1B) and samples from sample preparation (b) with mobile phases I and II (Figure 1A).

Measure peak heights of standard solution and samples.

Sodium saccharin in soft drinks,

$$\text{mg/L} = C' \times (H/H') \times (V'/V)$$

Sodium saccharin in sweets,

$$\text{mg/kg} = C' \times (H/H') \times (V'/V) \times 5.5$$

where C' = concentration of standard, mg/L; H and H' = average peak height of sample and standard, respectively; V and V' = volumes injected (μL) of sample and standard, respectively; and 5.5 = 5/10.00 × 110.0/10.00.

Results and Discussion

Eight collaborators reported results on soft drink and juice. Individual results, along with the recoveries and statistical parameters for each of the 6 samples, are shown in Table 1. Five collaborators reported results on sweets, and statistical parameters for the 3 samples are given in Table 2. Only approximate recoveries were shown for the samples of sweets.

The ranking test described by Youden and Steiner (3) showed the results of collaborator 3 to be outlying, and these were excluded from further analysis. Application of Dixon's outlier test to the individual results excluded the determinations of collaborator 6 for the level 1 juice sample and the determinations of collaborator 4 for the level 3 juice sample from further calculations.

A one-way analysis of variance showed that the differences between the laboratories included in the comparison were significant at the 1% level for all samples other than the level 2 sweet sample. The precision parameters for separate levels are presented in Tables 1 and 2.

A 2-way analysis of variance was performed to test laboratory–level interaction and to calculate pooled precision parameters separately for the juice, soft drink, and sweet samples. The laboratory–level interactions were statistically significant at the 1% level in all samples. The pooled precision parameters are presented in Table 3.

For the sweets, our own results agreed well with those of the collaborators. The mean results of the collaborators were 97–102% of those obtained in our laboratory. The exact amounts of saccharin in the sweets were unknown, but if the average recovery of the method is considered to be 89.6%, the results of the collaborators were rather near (80–102%) the declared amounts, which may be slightly exaggerated by the manufacturers.

No blind duplicates were included in the collaborative study, but some closely matched pairs were analyzed (3, 4). Juices 2 and 3 were considered to be closely matched pairs, because the sodium saccharin levels added were 80.0 and 85.0 mg/L; soft drinks 1 and 3 also contained similar concentrations of sodium saccharin, i.e., 67.0 and 50.0 mg/L.

Comments and Recommendations

Collaborators 1 and 8 suggested modifications of the step-gradient elution times and collaborator 1 suggested an alternative solvent for mobile phase I: 10% methanol and 90% acetic acid solution. Collaborator 6 was skeptical about the reproducibility of the step-gradient technique. If necessary, the times of the step-gradient elution and the concentration of methanol in mobile phase I can be varied according to the state of the LC column because column aging and other column parameters must be taken into account. Collaborator 6 questioned the retention times for saccharin and interfering materials such as preservatives. The retention times given in Figure 1 depend on the state of the column used. With step-gradient elution, interfering materials such as preservatives are not a problem (Figure 1A).

Collaborator 7 reported an investigation in which a phosphate buffer and methanol was used as a mobile phase and was preferred because it did not have the corrosive effect of acetic acid. However, this corrosive effect has not generally been observed. Statistical analysis of the results revealed no differences between these and all other results.

Collaborator 6 queried the most sensitive detection range. Detection at 254 nm was slightly more sensitive than detection at 280 nm with the fixed wavelength system, whereas the most sensitive wavelengths are 207 and 214 nm, which, at that time, were not generally in use. Collaborator 5 suggested the method could be improved by using fluorescence detection and reported that these results were in accordance with those obtained with UV detection at 254 nm. Additional comments from collaborator 5 indicated that the method was repeatable and that the preparation of the samples was time saving. This collaborator recommended the step-gradient elution for all samples.

Acknowledgments

The authors thank the following collaborators for their participation in this study:

Karen Berg-Nielsen, Oslo Board of Health Dept of Food Control, Oslo, Norway

Karin Blomberg, Finnish Customs Laboratory, Espoo, Finland

Merethe Guldborg, National Food Institute, Søborg, Denmark

Torunn Hagebø, The National Quality Control Authority for Processed Fruits and Vegetables, Oslo, Norway

Rolf Lundqvist, AB Findus, Bjuv, Sweden

Per Mattsson, National Food Administration, Food Research Dept, Uppsala, Sweden

Eeva-Liisa Niemi, Food and Environmental Laboratory of Helsinki, Helsinki, Finland

Toivo Savioja, Municipal Food Control Laboratory of Lahti, Lahti, Finland

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ALCOHOLIC BEVERAGES

Phosphorus in Wine: Comparison of Atomic Absorption Spectrometry Methods

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Phosphorus in wine may be determined directly or indirectly by atomic absorption spectrometry. The direct method uses the carbon rod atomizer as the excitation source and a phosphorus hollow cathode lamp. In the indirect determination, one measures the amount of molybdenum that will complex with phosphorus in the wine. Both nitrous oxide-acetylene and air-acetylene flames are suitable as atomization sources in this indirect method. The resultant data have been compared with those from the AOAC colorimetric method (11.032-11.034). A 2-sample comparison test showed the results to be insignificantly different at the 95% confidence limits.

Phosphorus has been determined by atomic absorption spectrometry in gasoline (1), edible oils (2), fertilizer (3), solid biological materials (4), starch (5), and rock samples (6). Most of these analyses used either the phosphorus electrodeless discharge lamp with the carbon rod atomizer for high sensitivity and low detection limits, an indirect method involving the measurement of molybdenum, or, in the case of samples with sufficiently high amounts of phosphorus, a phosphorus hollow cathode lamp with a nitrous oxide–acetylene flame (7).

Phosphorus in wine may combine with iron to form a ferric phosphate casse or cloudiness. This casse formation may adversely affect the sensory characteristics of the wine concerned. Phosphorus in wine generally has been measured indirectly by the AOAC colorimetric method (8). This method is time-consuming, requires the handling of hazardous acids, and requires the phosphorus to be in the orthophosphate form for sensitive detection. Interferences from other ions may also be a problem.

In this study we compared direct and indirect atomic absorption methods for the determination of phosphorus in wine with the AOAC colorimetric method:

Method I. AOAC **11.032-11.034**: (*a*) wet ashing technique; (*b*) dry ashing technique.

Method II. Direct atomic absorption spectrometry: phosphorus hollow cathode lamp (HCL), carbon rod atomizer as atomization source.

Method III. Indirect atomic absorption spectrometry: (*a*) molybdenum HCL, N_2O-C_2H_2 flame as atomization source; (*b*) molybdenum HCL, air-C_2H_2 flame as atomization source.

The direct method uses the carbon rod atomizer as the atomization source and a phosphorus hollow cathode lamp (9). This highly sensitive method requires minimal sample preparation and eliminates problems of flame absorption and flame noise. It is practically free from chemical interferences (6).

The indirect method is adapted from a Varian Techtron procedure (7) for the determination of phosphorus and silicon. It uses the molybdophosphoric acid complex as a means of extracting phosphorus from wine and the molybdenum hollow cathode lamp, rather than the phosphorus lamp, for absorbance measurements. Both nitrous oxide–acetylene and air–acetylene flames may be used as atomization sources.

Received October 15, 1985. Accepted May 15, 1986.

Experimental

Standards and Reagents

(a) *Commercial wine samples.*—Picked at random. Two red table wines and 2 white table wines were selected.

(b) *Phosphorus standard solution (Methods I and III).*—Prepare fresh weekly. Prepare phosphorus standard of 500 mg P/L by dissolving 2.2 g dried (2 h at 110°C) reagent grade potassium dihydrogen phosphate in water in 1 L volumetric flask. Store in amber glassware to prevent deterioration. Prepare working standards from master standard by dilution.

(c) *Phosphorus standard solution (Method II).*—Prepare fresh daily. Prepare phosphorus standard of 25 mg P/L with 0.2% Ni stabilizer by dissolving 0.0549 g reagent grade potassium dihydrogen phosphate (dried 2 h at 110°C) and 4.9535 g nickel nitrate hexahydrate in water and diluting to 500 mL in volumetric flask.

(d) *Molybdovanadate reagent (Method I).*—Dissolve 40 g ammonium molybdate tetrahydrate in 400 mL hot water. Dissolve 2 g ammonium metavanadate in 250 mL hot water. When this latter solution is cool, add 200 mL 70% perchloric acid and again cool. Add molybdate solution gradually to metavanadate solution with stirring and dilute to 2 L.

(e) *Molybdate reagent (Method III).*—Dissolve 25.00 g reagent grade ammonium molybdate in water and dilute to mark in 250 mL volumetric flask. Prepare this 10% w/v molybdate solution fresh weekly and use for the formation of molybdophosphoric acid. Store in amber glassware.

(f) *Basic buffer (Method III).*—Dissolve 53.30 g reagent grade ammonium chloride in water, add 70 mL ammonium hydroxide, and dilute to 1 L with water. Use this basic aqueous buffer solution for back-extracting molybdophosphoric acid from organic phase.

Table 1. Measured phosphorus content in wine samples as determined by several methods

Method	Phosphorus concentration, μg/mL[a]			
	White table wine	Red table wine	Chenin blanc	Burgundy
I. (a) Wet ash (AOAC)	189.7 ± 0.5	194.6 ± 2.6	135.5 ± 5.5	150.4 ± 5.6
(b) Dry ash (AOAC)	189.9 ± 5.9	189.9 ± 3.3	135.5 ± 14.7	147.4 ± 3.8
II. Phosphorus HCL with CRA	186.3 ± 10.1	195.0 ± 13.2	136.0 ± 8.50	146.7 ± 7.60
III. (a) Molybdenum HCL with N_2O-C_2H_2 flame	187.7 ± 2.1	192.1 ± 1.9	136.9 ± 1.3	148.0 ± 2.0
(b) Molybdenum HCL with air-C_2H_2 flame	187.7 ± 3.7	191.3 ± 2.5	135.4 ± 0.69	147.8 ± 1.0

[a] ± Standard deviation of 3 replicates.

Apparatus

Method I.—Spectronic 20 (Bausch and Lomb) and Pyrex cuvets for absorbance measurements at 400 nm. Samples may be wet-ashed in Pyrex glassware; use platinum crucibles and suitable muffle furnace for dry ashing.

Method II.—Atomic absorption spectrometer (Varian-Techtron Model AA-6) equipped with carbon rod atomizer, CRA-90, and phosphorus hollow cathode lamp. Pipet samples into graphite tube with adjustable micropipet (Oxford Laboratory, Model 1885-300106).

Method III.—Varian-Techtron atomic absorption spectrometer Model AA-6 (Varian Instrument Group, Sunnyvale, CA) with both nitrous oxide–acetylene and air–acetylene flame heads. Molybdenum hollow cathode lamp with resonance emission line at 313.3 nm as line source. Set the following instrumental parameters with each flame type:

Method III	*A*	*B*
Lamp current, mA	5	5
Fuel	acetylene	acetylene
Support	nitrous oxide	air
Flame stoichiometry	strongly reducing, red cone 2–3 cm	strongly reducing
Wavelength, nm	313.3	313.3
Spectral band pass, nm	0.2	0.2
Optimum working range, μg P/mL	2–16	50–160

Procedures

Method I.—See AOAC **11.032-11.034.** Prepare separate samples by wet-ash and dry-ash procedures and determine phosphorus as directed.

For series of wine samples, add 20 mL molybdovanadate reagent to each flask within 5 min interval, and dilute contents to mark. Turn on spectrometer and let it warm up 15 min before reading absorbance values at 400 nm. Use reagent blank to zero instrument and construct 4-point standard curve with 5, 10, 15, and 20 mg P/L solutions. During analyses, check standard in middle of working range of concentration every 5 samples. Determine absorbance value for each sample a minimum of 3 times and dilute samples, if necessary, to bring their absorbance values within linear range of calibration curve.

Method II.—Determine phosphorus in wine by using carbon rod atomizer, AA spectrometer, and phosphorus hollow cathode lamp. Use 213.6 nm wavelength at 20 mA lamp current and 1.0 nm spectral band width; do not use background correction. Operating parameters for CRA-90 instrument are as follows: dry, 100°C for 45 s; ash, 500°C for 5 s; atomize, 2500°C for 1 s; ramp, 800°C/s; sheath gas, nitrogen; stabilizer, 0.2% Ni (as nickel nitrate).

Construct 5-point calibration curve by injecting various (2, 4, 6, 8, and 10 μL) volumes of standard solution dispensed from adjustable micropipet. Run same volume of blank before each analysis to minimize background noise. Dilute all wine samples 1:20 with water; further dilute to 0.2% Ni concentration in 100 mL flask. Determine absorbance values for each wine a minimum of 3 times, using 5 μL injection volumes. Replace carbon tubes approximately every 50–60 injections and carbon electrodes approximately every 100 injections.

Method III.—Analyze phosphorus by extraction and AA spectrometry using molybdenum hollow cathode lamp as follows: Pipet working standards and 10 mL wine (or 1 + 9 diluted wine) into 125 mL separatory funnels and add 1 mL HCl (1 + 1) to each. Adjust total volumes to ca 50 mL with water. Add ca 4 mL ammonium molybdate solution and let solution stand 10 min. Add another 5 mL concentrated HCl, and let solution stand another 5 min. Add 45 mL diethyl ether and shake mixture vigorously at least 4 min. When 2 phases separate, discard lower aqueous phase and wash tip of funnel with water.

To remove excess molybdenum, add 10 mL HCl (1 + 9) and mix well with ether phase. Discard this acid layer and again wash tip of separatory funnel with water. Add 30 mL buffer solution, shake solution for precisely 30 s, and transfer aqueous layer to 50 mL volumetric flask. Add additional 15 mL buffer solution to ether layer and repeat process. Dilute combined aqueous layers to 50 mL with water and determine molybdenum absorbance of solution.

Construct 4- or 5-point standard addition curves for nitrous oxide–acetylene and air–acetylene flame methods, respectively. Determine phosphorus content of several wine samples from extrapolation of these standard addition curves. Measure absorbance values for each sample a minimum of 3 times over 3 s integration periods.

Table 2. Detection limits and characteristic amounts of various analytical methods for phosphorus in wine[a]

	Method	Detection limit[b]	Characteristic amount[c]	Sample size required, mL
I.	AOAC (wet ash and dry ash)	70.4 μg	15 μg	5 mL
II.	Phosphorus HCL with CRA	6.8 ng	5.2 ng	5 μL
III. (a)	Molybdenum HCL with N_2O-C_2H_2 flame	21.7 μg	1.3 μg	1.0 mL
(b)	Molybdenum HCL with air-C_2H_2 flame	145 μg	22 μg	10 mL

[a] Phosphorus content of typical wines ranges from 60 to 900 μg/mL.
[b] Detection limit calculated using 100 μg/mL standard. DL = $W(2S)/A$, where W = absolute amount of phosphorus, A = average of 10 absorbance values for this amount, and S = standard deviation of 10 absorbance readings.
[c] Characteristic amount is defined as amount of phosphorus required to produce signal of 0.0044 absorbance unit.

Results and Discussion

The results obtained from the various methods of analysis for phosphorus in several wines are listed in Table 1. Linear calibration curves were obtained in all cases. A statistical comparison of these results indicates no significant differences among them at the 95% confidence level. Detection limits and characteristic amounts are listed in Table 2 for each of the analytical methods.

The official AOAC wet and dry ash methods were included for the purpose of evaluating both the accuracy and precision of the alternative methods described. The colorimetric method measures only the orthophosphate form of phosphorus and therefore requires a lengthy sample preparation. The sample preparation time for the wet ash technique was over 1 h; over 8 h was required for the dry ash technique. A comparatively large sample size was also required. This official method is straightforward and easy to use. A Spectronic 20 spectrometer is commonly available and minimum training is required for its successful operation. The sample preparation procedures are also relatively easy to perform and could be readily followed by the analyst.

Phosphorus in wine cannot be directly determined by flame atomic absorption using a phosphorus hollow cathode lamp; the sensitivity is too low. Phosphorus may be directly determined, however, using the carbon rod analyzer as the atomization source. The normal working range with this technique is 1–200 μg P/mL with a detection limit of 0.0054 μg P. Therefore, wine samples should be diluted 1:20 for analysis. A band pass of 1.0 nm is used to further increase sensitivity.

Nickel is added at the 0.2% level in all samples to act as a modifier, increasing the vaporization temperature of the phosphorus and prolonging graphite tube lifetimes. When the phosphorus concentration exceeds 200 μg/mL, larger amounts of the nickel modifier must be used. When phosphorus concentrations are less than 50 μg/mL, the nickel stabilizer level should be reduced to one-half.

Sample preparation time is minimal in this technique, resulting in relatively fast analysis times. It takes fewer than 3 min to determine the phosphorus content in wine. All forms of phosphorus are measured and typical levels of dissolved solids in the diluted wine samples do not interfere. The CRA instrument is stable and straightforward to operate; however, it is a relatively expensive add-on to the basic AA unit. Its use requires the ability to pipet samples reproducibly into the graphite tube; this requires some technique and experience.

Determination of the molybdenum equivalent to phosphorus in wine involves an indirect approach with a molybdenum hollow cathode lamp and either the nitrous oxide–acetylene or air–acetylene flame. With the nitrous oxide–acetylene flame, wine samples are diluted 1:10 to get phosphorus levels within the normal working range of the standard curve. The detection limit under these conditions is about 22 μg P (1 mL of a 22 μg/mL sample) with a standard phosphorus solution, and the characteristic amount (mass of element giving a peak height of 0.0044 absorbance unit) is 1.3 μg P.

Although the standard addition method was used here to avoid matrix effects, indications are that it is not required. Both 0.5 nm and 0.2 nm spectral band passes have been investigated. They yield similar results; however, the 0.5 nm band pass produces greater background noise. The per sample preparation time is about ½ h, considerably less than that required in the official method. This time could be reduced further by use of an automatic shaker for the critical and time-consuming shaking process. The need to optimize fuel–oxidant ratios, burner position, and aspiration techniques with the nitrous oxide–acetylene flame requires a significant amount of training and experience prior to obtaining reproducible results.

An air–acetylene flame may also be used with the molybdenum hollow cathode lamp in another indirect approach to the determination of phosphorus in wine. Since this flame is less sensitive than the hotter nitrous oxide–acetylene flame, undiluted wine samples are used. A standard addition procedure was again followed resulting in a detection limit of 145 μg P (10 mL of a 14.5 μg/mL sample) and characteristic amount of 22 μg P.

This method is quite sensitive enough for determining the usual phosphorus levels found in wine. The air–acetylene flame is a more easily used and readily available atomization source. Its use is therefore preferable to that of the nitrous oxide–acetylene flame for wine analysis. Reproducible results again require a consistent aspiration technique, so a certain amount of operator training and experience is necessary.

Conclusion

Determination of phosphorus in wine can be successfully accomlished by any of the methods described above. The atomic absorption procedures have the advantage of requiring considerably less sample preparation. If one is processing large numbers of samples, the CRA attached to an autosampler would be an asset. With limited numbers of samples the molybdophosphate extraction procedure provides fairly rapid analysis times and requires only a conventional atomic absorption spectrometer. Either the nitrous oxide–acetylene or air–acetylene flames may be successfully used, with the latter being generally preferable.

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HAZARDOUS SUBSTANCES

Gas Chromatographic-Thermal Energy Analysis Method for Determination of Volatile *N*-Nitrosamines in Baby Bottle Rubber Nipples: Collaborative Study

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A collaborative study was conducted on the U.S. Food and Drug Administration (FDA) dichloromethane extraction method for determining volatile *N*-nitrosamines in baby bottle rubber nipples. Following dichloromethane extraction, *N*-nitrosamines were determined by gas chromatography-thermal energy analysis. Six pairs of blind duplicate rubber nipple samples representing 6 lots were analyzed by 11 collaborating laboratories. All samples were portions taken from equilibrated composites of cut-up rubber nipples obtained from manufacturers in the United States. Recoveries of the internal standard (*N*-nitrosodipropylamine) at approximately 20 ppb ranged from 10 to 120%. Reproducibility relative standard deviations (RSD_r) were between 35 and 45% for *N*-nitrosamine levels from 10 to 20 ppb. However, when data from laboratories with recoveries less than 75% were excluded (this is now specified in the method), RSD_r values were between 11 and 32% for *N*-nitrosamine levels from 6 to 26 ppb. Values were consistent with or better than those reported for other analytical techniques designed to quantitate trace contaminants at the low ppb level, e.g., aflatoxin in foods. The method has been adopted official first action for the quantitation of volatile *N*-nitrosamines in baby bottle rubber nipples.

Several reports have been published describing the presence of volatile *N*-nitrosamines in various rubber products (1, 2). These compounds, many of which are classified as carcinogens, are produced in rubber from the chemical accelerators and stabilizers added during the vulcanization process. The present concern about the occurrence of volatile *N*-nitrosamines in baby bottle rubber nipples and the possible migration of these compounds into infant formula was prompted by a report of Preussmann et al. (2) in which the investigators, using an aqueous extraction technique, found *N*-nitrosamines at levels up to 230 parts per billion (ppb) in all 70 rubber samples that they analyzed. Havery and Fazio (3) developed a dichloromethane extraction method for this analysis. In a preliminary study, they reported the presence of 3 *N*-nitrosamines at levels up to 281 ppb in 2 types of nipples from one manufacturer. Havery and Fazio (4) also conducted a local market survey of 27 baby bottle nipples and found *N*-nitrosodimethylamine (NDMA), *N*-nitrosodiethylamine (NDEA), *N*-nitrosodibutylamine (NDBA), and *N*-nitrosopiperidine (NPIP) levels up to 387 ppb. All 4 *N*-nitrosamines migrated from the rubber nipples into water, milk, and infant formula when sterilized together in a manner typical of that used in the home.

On January 1, 1984, the U.S. Food and Drug Administration (FDA) established an action level of 60 ppb total *N*-nitrosamines in rubber nipples. The action level was reduced to 10 ppb on January 1, 1985. A collaborative study was conducted on the dichloromethane extraction method of Havery and Fazio (3) for volatile *N*-nitrosamines in baby bottle rubber nipples to determine the suitability of the method to support the 10 ppb *N*-nitrosamine action level.

Submitted for publication August 4, 1986.
This report of the Associate Referee, J. I. Gray, was presented at the 100th AOAC Annual International Meeting, Sept. 15–18, 1986, at Scottsdale, AZ.
The recommendations of the Associate Referee were approved by the General Referee and the Committee on Foods I and were adopted by the Association. See the General Referee and Committee reports, *J. Assoc. Off. Anal. Chem.* (1987) **70**, March issue.
Michigan Agriculture Experiment Station Journal Article No. 11738.

Collaborative Study

Eleven collaborating laboratories analyzed 6 pairs of blind duplicate rubber nipple samples representing 6 lots. All samples were taken from equilibrated composites of rubber nipples obtained from major manufacturers in the United States. For the collaborative study, nipples were cut up and allowed to equilibrate for 1 week in large glass jars at room temperature before aliquots were removed for the individual laboratories. This equilibration was considered necessary to ensure uniform samples for analysis, in contrast with the common practice of immeditely analyzing nipples after they have been cut into small pieces (3).

Collaborators were instructed to make at least one practice run to become familiar with the method and then to perform the determination according to the specified sampling schedule, because delay might cause the samples to deteriorate. They were also instructed to make only the number of determinations requested by the Associate Referee (one analysis per sample) and to report all results. *N*-Nitrosamine standard solutions were provided for each collaborator.

N-Nitrosamines in Baby Bottle Rubber Nipples

Gas Chromatographic Method

First Action

Principle

Volatile *N*-Nitrosamines are extd from cut-up rubber nipples with CH_2Cl_2. Soln is made alk., CH_2Cl_2 and H_2O are distd, and *N*-nitrosamines in aq. phase are partitioned back into CH_2Cl_2. Ext is concd for detn by gas chromatgy with thermal energy analysis.

Apparatus

(a) *Soxhlet extractor.*—40 mm extn tube with connections for ☨ 45/50 Allihn condenser and ☨ 24/40 250 mL flask. Extn thimble, borosilicate glass, 80 × 33 mm, 30 mL capacity, with coarse porosity frit.

(b) *Evaporative concentrator.*—Kuderna-Danish, 250 mL, with ☨ 24/40 column connection and ☨ 19/22 lower joint; size 425 concentrator tube, 4 mL capacity, with ☨ 19/22 joint; 3-ball Snyder distg column with ☨ 24/40 joint.

(c) *Filtering funnel.*—60 mL with coarse porosity frit.

(d) *Gas chromatograph.*—Hewlett-Packard 5710A, or equiv., interfaced with Model 502L thermal energy analyzer (Thermedics, Inc., 470 Wildwood St, PO Box 2999, Woburn, MA 01888), or equiv. GC-TEA conditions: 2.7 m × 4 mm id glass column packed with 10% Carbowax 1540/5% KOH on 100–120 mesh Chromosorb W(HP) (Supelco). Argon carrier gas flow at 40 mL/min. Temps: column programmed from 100 to 180° at 4°/min; injection port 200°; TEA furnace 450°. Adjust attenuation according to sensitivity of instru-

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Table 1. Total *N*-nitrosamine levels (ppb, uncorrected for recovery) in 6 blind duplicate pairs of rubber nipple samples representing 6 nipple lots[a]

Lab.	Lot 1		Lot 2		Lot 3		Lot 4		Lot 5		Lot 6	
1	14	17	9	11	0	0[b]	11	12	88	63	11	12
	(88)	(67)	(88)	(97)	(78)	(58)	(84)	(72)	(80)	(59)	(75)	(59)
2	15	17	12	11	0	0	12	16	37	16	15	14
	(90)	(83)	(81)	(83)	(93)	(90)	(88)	(93)	(95)	(76)	(90)	(93)
3	15	17	12	11	1	1	14	14	20	20	13	14
	(82)	(89)	(90)	(76)	(93)	(85)	(94)	(86)	(83)	(87)	(95)	(95)
4[c]	47	45	12	13	6	6	125	123	45	43	115	147
	(65)	(69)	(83)	(75)	(67)	(91)	(78)	(93)	(75)	(67)	(88)	(93)
5	9	6	7	5	3	4	11	6	11	1	6	7
	(73)	(55)	(94)	(83)	(56)	(66)	(83)	(80)	(60)	(15)	(70)	(84)
6	25	18	13	20	1	1	15	17	41	23	14	15
	(93)	(80)	(78)	(78)	(10)	(10)	(69)	(67)	(61)	(80)	(70)	(66)
7	24	24	10	10	3	4	15	11	26	22	14	15
	(95)	(81)	(78)	(99)	(84)	(80)	(96)	(78)	(68)	(96)	(78)	(80)
8	12	18	16	18	4	4	17	15	38	29	18	14
	(57)	(78)	(105)	(75)	(83)	(98)	(99)	(53)	(77)	(96)	(66)	(59)
9	15	16	9	10	1	2	15	23	4	19	22	16
	(82)	(83)	(109)	(107)	(38)	(35)	(121)	(100)	(20)	(83)	(115)	(88)
10	31	29	13	69	0	0	21	20	23	19	6	10
	(83)	(65)	(93)	(99)	(72)	(84)	(71)	(66)	(83)	(74)	(65)	(100)
11	17	15	8	6	0	0	15	16	29	29	15	16
	(66)	(73)	(83)	(41)	(32)	(39)	(93)	(104)	(76)	(89)	(83)	(98)

[a] Numbers in parentheses represent percent recovery of internal standard (NDPA) for each analysis.
[b] Zero *N*-nitrosamine = none detected.
[c] Data from Laboratory 4 not included in statistical analyses. Laboratory did not adhere to analytical protocol.

ments (8 μL injection of *N*-nitrosodipropylamine (NDPA) internal std soln E should give minimum GC-TEA response of 50 mm).

(e) *Trap.*—Liq. N.

Reagents

(a) *Dichloromethane.*—Distd in glass.

(b) *Water.*—Double distd.

(c) *N-Nitrosamine std solns.*—All solns prepd in CH_2Cl_2. For each suspected *N*-nitrosamine, prep. sep. stock soln (A) by dissolving 100 mg in 10 mL CH_2Cl_2. Dil. 1 mL of each soln A to 100 mL (soln B, 100 μg/mL). Pipet 1.0 mL of each soln B into single vol. flask and dil. to 200 mL (combined soln C, 0.5 μg of each *N*-nitrosamine/mL). Dil. 2 mL soln C to 10 mL (soln D, 0.1 μg of each *N*-nitrosamine/mL). Dil. 10 mL stock soln of NDPA (soln B) to 100 mL and further dil. 1 mL of this soln to 100 mL (internal std soln E, 100 ng NDPA/mL). Soln E may be prepd from com. dild std.

Caution: *N*-Nitrosamines are potent animal carcinogens and must be handled with appropriate safety precautions.

Determination

Blank test.—To ensure absence of interfering peaks, run sep. blank tests on CH_2Cl_2 and H_2O. Also perform total reagent blank to check all reagents. Check CH_2Cl_2 by concg 200 mL to 0.5 mL in Kuderna-Danish app. and injecting 8 μL aliquot into GC-TEA system. Check H_2O by partitioning 100 mL H_2O with three 50 mL portions of CH_2Cl_2. Pass through anhyd. Na_2SO_4 to dry, conc. to 0.5 mL in Kuderna-Danish app., and inject 8 μL aliquot into GC-TEA system. If interferences occur, discard. Test CH_2Cl_2 for potential to nitrosate amines by adding 100 mg morpholine to 100 mL solvent and holding overnight at room temp. Conc. to 1 mL and perform GC-TEA analysis. Little or no *N*-nitrosomorpholine should be observed.

Extraction.—Place 5 g cut-up sample in 250 mL g-s r-b flask, add 100 mL CH_2Cl_2, stopper flask, and hold overnight (17–18 h). Attach 250 mL flask containing boiling chips to Soxhlet extn app. Quant. transfer CH_2Cl_2 ext and nipple pieces thru funnel to extn thimble in Soxhlet app. Wash flask with two 12 mL portions of CH_2Cl_2 and add washings to extracator. Spike soln in Soxhlet app. with 1.0 mL NDPA internal std soln E and attach to H_2O-cooled condenser. Attach heating mantle to flask and ext 1 h at Variac setting of 85% (102 V). Remove heating mantle and let extractor cool 15 min. Siphon any CH_2Cl_2 remaining in Soxhlet app. into flask.

Distillation.—To CH_2Cl_2 ext add boiling chips, 100 mL 5N NaOH, and 2 g $Ba(OH)_2$. Attach to atm. distn app. Carefully distil CH_2Cl_2 at Variac setting of 30% (36 V). Discard all CH_2Cl_2 distillate. Adjust Variac setting to 71% (86 V) and collect 70 mL aq. distillate in calibrated 250 mL sep. funnel.

Liquid-liquid partition.—Add 300 mg anhyd. Na_2CO_3 to distillate, then add 50 mL CH_2Cl_2, and shake vigorously 1 min; let org. and aq. layers sep. Repeat this extn twice. Combine CH_2Cl_2 exts in 250 mL sep. funnel. Then pass combined exts through 30 g anhyd. granular Na_2SO_4 (held in 60 mL filtering funnel, (c), and pre-washed with 25 mL CH_2Cl_2) into 250 mL Kuderna-Danish evaporator. Wash Na_2SO_4 with 15 mL CH_2Cl_2 and add wash to Kuderna-Danish app.

Concentration of extract.—Add 2 or 3 Carborundum grains to flask, attach 3-ball Snyder column, and carefully conc. ext at rate of 1 mL/min, to 4 mL in 60° H_2O bath. Remove Kuderna-Danish app. from bath and let cool 15 min. Remove concentrator tube and carefully conc. ext to 1.0 mL under gentle stream of N. (Note: To facilitate concn of oily samples, immerse tip of concentrator tube in beaker contg H_2O at ca 40°.) Stopper tube, and retain for GC-TEA analysis.

GC-TEA analysis.—Sep. inject 8 μL *N*-nitrosamine std soln D and NDPA internal std soln E, and carry out chromatgc analyses. Using same conditions, inject 8 μL concd sample ext and carry out chromatgc analysis. Repeat injection sequence for *N*-nitrosamine std soln, NDPA internal std soln, and sample ext. Measure peak response of *N*-nitrosamines in std and sample exts that occur at same retention time.

Calculations

$$\text{N-nitrosamine in sample, } \mu g/kg = (C_{std} \times V \times P_{sp} \times 1000)/(P_{std} \times S_{wt})$$

$$\text{NDPA recovery, \%} = (P_{sp} \times 100)/P_{std}$$

where V = vol. sample ext (1.0 mL); C_{std} = std concn (0.1 μg/mL); P_{sp} = peak response of sample; P_{std} = peak response of std; S_{wt} = sample wt, g. Report results to nearest 0.1 μg/kg (ppb). Reject data with internal std rec. <75% and re-analyze sample.

Table 2. *N*-Nitrosodimethylamine levels (ppb, uncorrected for recovery) in 6 pairs of blind duplicate rubber nipple samples representing 6 nipple lots

Lab.	Lot 1		Lot 2		Lot 3		Lot 4		Lot 5		Lot 6	
1	10.4	17.2	0	1.9	0	0	1.4	1.1	87.6	63.1	0	0
2	11.7	11.7	0	0	0	0	1.1	1.6	37.4	15.5	0.8	0.8
3	12.4	14.0	1.2	1.0	0.9	0.9	1.9	1.9	20.1	19.9	1.5	1.7
4*	45.3	44.6	2.4	1.5	1.0	3.8	0	4.5	45.1	41.0	0	0
5	7.1	4.4	0.5	0.6	0.8	1.4	0.8	0.4	10.5	1.4	0.6	0
6	24.1	14.9	14.9	4.1	1.4	1.1	2.3	1.0	31.8	22.5	0.6	0.8
7	21.3	20.9	0.9	1.0	0.6	0.5	1.8	1.2	26.2	21.7	1.0	1.2
8	9.4	15.0	4.1	1.8	1.6	1.0	2.6	1.8	35.9	28.5	1.6	1.3
9	13.1	13.9	0	0	0	0	1.9	4.7	0	18.7	0	0
10	31.0	28.5	2.9	48.2	0	0	1.0	0.8	22.8	19.2	0	0.8
11	14.3	14.7	0	0	0	0	2.0	2.0	29.2	28.6	2.5	1.1

* Data from Laboratory 4 not included in statistical analyses (see Table 1).

Results and Discussion

Eleven laboratories concerned with the analysis of *N*-nitrosamines in baby bottle rubber nipples participated in the collaborative study. A preliminary study included all participating laboratories to determine competence with the method. Results of duplicate analyses of the 2 preliminary nipple samples indicated that all laboratories were reasonably familiar with the method, but that some laboratories required further familiarization.

In the collaborative study, 6 pairs of blind duplicate rubber nipple samples (representing 6 nipple lots) were analyzed as homogeneous nipple pieces rather than whole nipples. This was done to minimize, as far as possible, the expected *N*-nitrosamine level variation resulting from within-lot differences of individual nipples. *N*-Nitrosamine data submitted by the 11 laboratories are tabulated in Tables 1–4. These data are uncorrected for internal standard recovery for several reasons. First, a wide range of recoveries was reported by the collaborators. This was due both to lack of experience with the method and to instrumentation problems encountered by some of the less experienced laboratories. Also, *N*-nitrosamine data are historically not corrected for internal standard recovery because of differences in volatility among *N*-nitrosamines. An internal standard was used solely to maintain a check on the performance of the method, so the data for samples with less than 75% recovery of the internal standard were not statistically evaluated. This criterion is now included in the method.

Several laboratories reported low levels (generally lower than 2 ppb) of other *N*-nitrosamines (NDBA, NPIP) in some of the samples, which other collaborators did not observe, because of failure to decrease instrument attenuation. However, the reported values are included in the data on total *N*-nitrosamines in Table 1. Instructions regarding measurement of low levels of *N*-nitrosamines have been added to the method.

The data were statistically analyzed according to Youden and Steiner (5). These analyses were carried out independently by statisticians from both Michigan State University and FDA. The results from Laboratory 4 were not included in the statistical analyses because of deviations from the method, as evidenced by the report. Data for Laboratory 5 were rejected on the basis of instrumental problems, as verified by the Youden rank sum. Youden's procedure (5) was used to test for laboratories that reported values either consistently higher or lower than the other laboratories. From critical value tables (5), laboratories with a rank sum greater than 57 (Laboratory 4 = 62) or less than 15 (Laboratory 5 = 13.5) could be rejected at the 5% level. Laboratory 10 was rejected on the basis of a report submitted by the collaborator that use of inferior quality solvents caused *N*-nitrosamine artifact formation. Evidence for this problem can be seen in data submitted by Laboratory 10, Lot 2 (Table 1), and in the wide range of internal standard recoveries (65–100%).

We examined the "repeatability" (RSD_o; previously, CV_o) and "reproducibility" (RSD_x; previously, CV_x) of the reported results. The examination focused on NDMA, NDEA, *N*-nitrosomorpholine (NMOR), and total (uncorrected) *N*-nitrosamine values. RSDs of those *N*-nitrosamines found at low levels by some collaborators were not calculated because of the large bias that would result from the presence of many zero values.

Examination of the reproducibility relative standard deviation (hereafter referred to as reproducibility) and the repeatability relative standard deviation (hereafter referred to as repeatability) on a lot-by-lot basis is shown in Table 5. Outlying laboratories were detected by the 3 methods de-

Table 3. *N*-Nitrosodiethylamine levels (ppb, uncorrected for recovery) in 6 pairs of blind duplicate rubber nipple samples representing 6 nipple lots

Lab.	Lot 4		Lot 6	
1	10.0	10.8	10.5	11.6
2	11.3	14.4	14.4	12.8
3	12.2	12.1	11.2	11.8
4*	125.0	118.2	114.6	146.9
5	10.0	5.2	5.1	6.6
6	10.9	10.8	10.9	10.9
7	13.2	9.6	12.7	12.8
8	13.4	12.5	10.0	13.1
9	13.0	17.9	12.9	15.6
10	19.8	18.7	6.1	10.3
11	13.1	12.4	12.8	12.9

* Data from Laboratory 4 not included in statistical analyses (see Table 1).

Table 4. *N*-Nitrosomorpholine levels (ppb, uncorrected) in nipple lot 2 (blind duplicate samples 2 and 8)

Lab.	Lot 2	
1	4.8	5.7
2	5.7	5.8
3	8.1	6.8
4*	6.9	6.3
5	3.9	3.0
6	5.4	14.1
7	6.1	6.6
8	8.5	6.1
9	5.8	5.8
10	6.0	8.5
11	6.4	5.8

* Data from Laboratory 4 not included in statistical analyses (see Table 1).

Table 5. *N*-Nitrosamine data (uncorrected) reported by 8 laboratories for 6 nipple lots—calculated values for repeatability (RSD_o), reproducibility (RSD_x), and historically expected reproducibility (RSD_x)[a]

Nipple lot	*N*-Nitrosamine	$\bar{X}$, ppb	RSD_o, %	RSD_x, %	Expected RSD_x, %
1	NDMA	14.1	5.5	27.0	30.4
	NMOR	2.3	42.9	42.9	39.8
	Total	18.2	13.3	22.2	29.2
2	NMOR	6.4[b]	15.5	16.3	33.9
	Total	12.0	17.3	29.3	31.1
3	Total	1.9	18.7	102.7	41.1
4	NDMA	2.1	50.3	52.2	40.5
	NDEA	12.7	17.1	17.1	30.9
	Total	14.9	20.9	21.5	30.1
5	NDMA	24.6[c]	30.8	30.8	28.0
	Total	25.6[d]	31.5	31.5	27.8
6	NDEA	12.7	7.9	11.1	30.9
	Total	14.8	13.5	18.7	30.2

[a] Laboratories 4, 5, and 10 and data with NDPA recovery <75% omitted. See text.
[b] Laboratory 6, Cochran outlier, omitted.
[c] Laboratory 1, Dixon and Grubbs outlier, omitted.
[d] Laboratory 1, Gibbs outlier omitted. Laboratory 2, Cochran outlier; data retained to maintain 92% of the data points.

scribed by Grubbs and Beck (6), Dixon (7), and Cochran (8). Included in Table 5 are the RSD_x values historically expected at these *N*-nitrosamine concentrations as described by Horwitz et al. (9).

The major focus of this study was determination of the reproducibility of the method for quantitation of *N*-nitrosamines in rubber nipples at the 10 ppb level. An examination of the data shows that the method performance agrees favorably with and in almost all cases is better than that expected for contaminants at this level. The method is not reproducibile at *N*-nitrosamine levels of 2 ppb or less.

Comments of Collaborators

The collaborators were encouraged to submit any comments, suggestions, criticisms, or description of difficulties pertaining to the method which they considered important. The following is a summary of their responses.

(*1*) Certain laboratories indicated that they would like to see a "system suitability test" added to the method to ensure that all laboratories obtained adequate separation of the *N*-nitrosamines and to ensure reproducibility and linearity of detector response. Because all participating laboratories were routinely using the method to determine *N*-nitrosamine levels in rubber nipples, and the results of the preliminary study indicated competence with the method, we concluded that such a test was not necessary in this instance. In addition, the injection sequence used in the collaborative study (*N*-nitrosamine standard, internal standard, sample, repeat sequence) for each sample also ensured that the chromatographic separation and technique were adequate and uniform.

(*2*) The major criticism of the method was the use of a standard containing 0.5 μg of each of the 7 *N*-nitrosamines per mL dichloromethane. It was recommended that the method be performed with a standard mixture solution at concentrations expected in the nipple samples. It was also recommended that the concentration of the internal standard *N*-nitrosamine spiking solution be similar to that of the *N*-nitrosamine standard mixture. A standard solution containing 0.1 μg of each *N*-nitrosamine per mL was suggested. Such a concentration would permit standard and sample extracts to be run at the same instrument attenuation, thus eliminating any concern over linearity of detector response. We observed during this study that several laboratories ran sample extracts, internal standard, and the standard *N*-nitrosamine mixture at an attenuation setting that permitted the standard *N*-nitrosamine peaks to remain on scale. Consequently, these laboratories failed to detect the presence of *N*-nitrosamines in rubber nipples at 2 ppb or less. The method has been modified to incorporate the suggestion about standard *N*-nitrosamine concentrations.

(*3*) Some laboratories raised the question of the problem of peak detection. What guidelines/recommendations are available for determining whether a peak should be considered to be due to an *N*-nitrosamine? It was pointed out that because of the sensitivity of the method, the inclusion of several "unknown" peaks, i.e., those not corresponding to any of the 7 *N*-nitrosamines in the standard mixture, could make a difference of about 5 ppb, half the proposed 1985 limit. The method has been modified to state that only those peaks corresponding to the *N*-nitrosamine standards be measured.

(*4*) There was some criticism that the method did not evaluate expected *N*-nitrosamine level variation resulting from within-lot differences of individual nipples, because collaborating laboratories were supplied with homogeneous nipple pieces rather than whole nipples. It was to attain homogeneity (as far as reasonably possible) that the nipple pieces were equilibrated for 1 week at room temperature before they were sent to the individual laboratories.

(*5*) Two further comments were directed at the samples used in this study: (a) A small number of the laboratories reported very low recoveries (10–30%) of the internal standard (NDPA) for several of the test samples. A collaborator suggested that these low recoveries could be the result of a matrix effect from nipples produced by a particular manufacturer. However, the majority of the participating laboratories did not observe this matrix effect. (b) Another collaborator suggested that nonuniformity (hot spots) of samples may occur in certain instances. This might explain the small variations in *N*-nitrosamine levels in paired duplicate samples or the different results obtained when a collaborator was asked to repeat the analysis of a particular sample.

(*6*) Some general comments and recommendations pertaining to the details of the method were received. These include: (a) using smaller pieces of nipple (2 × 2 mm) to ensure a more complete extraction of the *N*-nitrosamine; (b) gently shaking the sample overnight after the addition of dichloromethane in a wrist-action shaker to ensure a more complete extraction of the *N*-nitrosamine; (c) recovering dichloromethane from the distillation step and using it to extract the *N*-nitrosamines from the aqueous distillate; (d) using a graduated measuring cylinder to collect the aqueous distillate (70 mL) instead of a 250 mL separatory funnel; (e) using a 3-ball micro-Snyder condenser instead of a stream of nitrogen in the final concentration step; and (f) eliminating or modifying the use of alkaline NaOH in the atmospheric distillation step, because alkaline solutions corrode glassware. Other comments concerned the type of gas chromatograph used, model of the thermal energy analyzer, length of the chromatographic column (glass vs stainless steel), and type of rheostat used in the refluxing and steam-distillation steps.

(*7*) One collaborator suggested that propyl gallate, a nitrosation inhibitor, should be added to the dichloromethane and nipple sample during nitrosamine extraction. Following the study, it was determined that this collaborator obtained

highly variable results due to *N*-nitrosamine artifact formation because of the use of poor quality dichloromethane containing nitrosating agents. Dichloromethane should first be checked for nitrosating potential by adding 100 mg morpholine to 100 mL solvent and holding at room temperature overnight. Gas chromatographic-thermal energy analysis of the concentrated solvent should result in little or no observable NMOR. It has been suggested that poor quality dichloromethane can be improved by partition with alkali and/or passage through an alumina chromatography column. The Associate Referee suggests that only dichloromethane that meets the above test be used.

Recommendations

It is recommended:

(*1*) That the method be adopted official first action for the quantitation of *N*-nitrosamines in baby bottle rubber nipples.

(*2*) That proficiency testing be conducted periodically (at least quarterly) to ensure that all laboratories involved in nipple analyses obtain consistent and reproducible *N*-nitrosamine values. It is also recommended that further studies be conducted with standard *N*-nitrosamine solutions to ascertain where the major problems with recovery of the internal standard occur.

(*3*) That results reported with an internal standard recovery of less than 75% be rejected. Recoveries of greater than 80% are recommended to ensure more accurate *N*-nitrosamine data. However, the internal standard should be used only as a means of evaluating competence with the method.

(*4*) That laboratories become familiar and proficient with the method before using it on a routine basis. This would ultimately provide accurate data for in-house evaluation of nipple products.

Acknowledgment

The following individuals participated in the collaborative study:

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SYMPOSIUM ON CRITICAL ANALYSIS OF ANALYTICAL METHODS FOR MEAT FOODS

99th Annual International Meeting of AOAC, October 1985

Meat foods are a principal component of the American diet. The U.S. Department of Agriculture has the responsibility of ensuring to the consumer the safety and quality of the meat and meat products under Federal inspection. Recent changes in processing procedures and developments in food technology for animal-based foods and an increased public interest in the composition of such foods have required USDA to examine and often to develop or modify analytical methods that characterize fresh and processed meat products to support inspection and labeling requirements.

This symposium was organized jointly by the Agricultural Research Service (ARS) and the Food Safety and Inspection Service (FSIS) divisions of USDA to provide a review of the adequacy of the analytical methodology for meat foods and an update on detection of permitted or proposed meat additives. The presentations examined AOAC official methods and other analytical procedures that have been used with meat products, particularly the nitrogen, protein, lipids, and amino acid methods; the development and examination of more rapid analytical methods for meat composition; and the present state of methods for characterizing soy protein and fish protein concentrates that may be added either legally or illegally. This symposium provided an indication of the present state of analytical methodology in meats, and, we hope, may stimulate research in view of changing technology to provide additional rapid, accurate procedures to characterize new products.

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Determination of Nitrogen and Protein Content of Meat and Meat Products

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Chemical and instrumental methods for determination of nitrogen and protein are reviewed for their mode of action and utility in analysis of meat proteins and products. Although the Kjeldahl digestion method is satisfactory for determining total nitrogen, it is imprecise for determining total protein content. Presence of variable amounts of nonprotein nitrogenous components and of connective tissue proteins such as collagen and elastin produces error if the formula (N × 6.25) is used to calculate crude protein. Such fibrous proteins have higher nitrogen levels (over 18%) than other muscle proteins (about 16%), and a higher than actual protein value will be determined unless a lower conversion factor is used to correct for their content. To determine meat protein content more accurately, a combination of Kjeldahl determination with one or more additional tests to correct for nonprotein and fibrous protein content is recommended. The choice of the additional method(s) is based on the user's requirement for protein characterization, available time, type of meat product, and sample size.

During the last decade, the U.S. Department of Agriculture has conducted research on meat and meat products to answer concerns of various consumer groups regarding the nutritional and health aspects of animal-based products. Specific concerns include the level and types of proteins and fats, as well as the value of the cure additives salt and nitrite. Consumers want an indication of the compositional levels in these products as purchased. With fresh meat products such as steaks, chops, and roasts, the amount of lean or fat tissues usually can be seen by the market shopper. With ground fresh meats and processed meats, however, variable amounts of fatty and connective tissues and nonmeat additives could be included without obvious changes in overall appearance. Consequently, legislation and analytical methods have been required to define and guarantee nutritional quality for such products.

Meat is defined in the Federal Register (1) as "any edible portion of the carcass of any cattle, sheep, swine, or goats, exclusive of lips, snouts, ears, caul fat, leaf fat, kidney fat, and other visceral fat, and exclusive of all organs, except the heart, tongue, and esophagus." Skeletal meat is "skeletal muscle tissue with accompanying fat that has been attached directly to bone, including that from the diaphragm and cheeks after they are trimmed to remove glandular tissue." Definitions for meat for the European market (2) are similar and

Accepted March 24, 1986.
Presented at the Symposium on Critical Analysis of Analytical Methods for Meat Foods, 99th AOAC Annual International Meeting, Oct. 27–31, 1985, Washington, DC.

include the animals listed above. Both sets of definitions for meat refer to the muscle tissue and associated connective tissues of domesticated animals and do not include the edible flesh from poultry, seafood, or game (1, 2).

Such definitions for meat are for source and location and do not contain compositional analysis. The gross composition for moisture, fat, and protein of various adipose and skeletal muscle tissues used in meat processing (Table 1) vary from lows of 1% protein and 8% water to 20% and 70%, respectively. In the most general sense, the combination of moisture and protein in meat constitutes the lean portion.

The wide variability in composition of the known components and the presence of uncharacterized substances in meats of unknown history prevent a strict characterization by defined content. The interrelationship of the compositional components in fresh meats with linear correlation coefficients generally greater than 0.95 (Table 1) allows use of predicted values for multi-analysis instrumental methods. For many skeletal tissues, moisture and protein vary in direct proportion, while fat content is inversely proportional to the other 2 components. Muscle proteins can be separated in fresh unheated meats on the basis of solubility in aqueous solutions of low or high ionic strength into 3 fractions—sarcoplasmic, myofibrillar, and stromal (3). When meat tissues are processed or heated, however, such fractionation methods are not usable because of heat denaturation and chemical interactions.

Table 1. Composition of typical meat processing materials[a,b]

Material	Protein, %	Fat, %	Moisture, %
Fat pork	0.9	91.7	8.1
Skinned jowls	7.5	71.2	20.9
Pork trimmings 50/50	9.1	54.5	35.9
Pork cheek meat	17.2	21.8	60.2
Beef chuck meat	18.3	11.4	69.4
Fresh bull meat	20.1	8.8	70.1

[a] Source of data: C. E. Swift, U.S. Dept of Agriculture, ERRC, Philadelphia, PA, personal communication, 1980.

[b] Linear correlation coefficents for these data: protein : fat, $r = -0.993$; protein : moisture, $r = 0.988$; fat : moisture, $r = -0.999$.

Analytical Methods for Nitrogen and Protein

Stewart (4) has indicated that the state of development of analytical methods in food products can be considered *adequate* for total nitrogen, *substantial* for most amino acids, and *conflicting* for total protein and some amino acids. Because proteins are an important part of human nutrition, various methods have been developed for their determination. These procedures make use of specific components of the proteins, i.e., peptide linkages, amino groups, selected amino acids, or attached carbohydrates, to quantitate protein content. Those methods that have been applied to determination of protein content of meat and meat products can be divided into 10 classes: (a) nitrogen content, (b) peptide linkages, (c) primary amino groups, (d) aromatic amino acids, (e) basic amino acids, (f) imino acids, (g) sulfur amino acids, (h) amides, (i) carbohydrates, and (j) "crude" dry protein. Some of these have been adopted at various times as AOAC official methods, but most have been developed or used only for specific purposes such as estimation of connective tissue proteins. Each of the 10 classes of analytical determinants will be examined for its applicability to meat studies. Depending on the type of protein and the source, widely divergent values for protein content can be obtained from the application of these analytical methods to the same sample.

(a) *Nitrogen content.*—Determination of total nitrogen and conversion to protein content by an appropriate factor is the basis for 4 AOAC official methods (5) for protein measurement in meat products. These methods require that the sample be a food and that nitrogen not be present in significant amounts as nitrate, or as azo or nitroso groups. Method **24.027** refers to the standard Kjeldahl method, **2.057**. In this procedure, the sample is digested at high temperatures with concentrated sulfuric acid, sodium sulfate, a metallic catalyst, and sometimes hydrogen peroxide to convert nitrogenous substances to ammonium salts. Addition of concentrated alkali to this digest converts the ammonium salts to free ammonia which is distilled, collected, and titrated by various procedures. Two other methods (**24.028–24.036** and **24.037**) use the basic Kjeldahl digestion with variations in the determination of ammonia nitrogen to allow automation and increased determinations per time period. The first of these methods uses a common sample digestion with provisions for both phosphorus and nitrogen determinations. Ammonia nitrogen is measured by reaction with hypochlorite and phenate ion in alkali to produce a quinonechloramine which reacts further with phenate to produce indophenol, which can be measured spectrophotometrically at 630 nm. The second method refers to **7.021–7.024** wherein the sample is digested by the Kjeldahl method and the ammonia produced is steam distilled and determined by an automated procedure. These 3 methods are defined for determination of *total nitrogen.* Percent nitrogen is calculated from milliequivalents ammonia per g sample by multiplying by the factor 1.4007. This value can be converted to crude protein content by a second factor: 6.25. A recent AOAC method and apparently the only specific listing for *crude protein* in meat is the block digestion method (**24.038–24.040**) which is not automated, but which allows a greater number of determinations to be conducted per unit of time. This method also uses the standard Kjeldahl digestion followed by steam distillation and titration, and conversion with the 2 factors listed previously. Whereas the Kjeldahl method is recognized as accurate and precise for nitrogen determinations (4), its application for protein content in certain products has been questioned because of the value of the conversion factor. AOAC method **14.067** (5) states that "Protein = nitrogen × 6.25 except for wheat and its products in which protein = N × 5.7. For all analyses, report %N and conversion factor used. Other traditional and customary factors are 5.18 for almonds, 5.46 for peanuts and brazil nuts, 5.30 for tree nuts and coconuts, and 6.38 for dairy products." In certain other products such as organ meats which contain large quantities of nucleic acids, a correction must be made for their contribution to total nitrogen. Deoxyribonucleic acids (DNA) contain 16% nitrogen, and a considerable error would be introduced in terms of protein content. The apparent value for protein would therefore be inflated by the additive nitrogen quantities from the protein and DNA content.

Other methods have been developed to determine nitrogen content by a variation of the Dumas method (5) (**6.016–7.020**), which has been applied for analysis of animal feeds. In these methods, the sample is combusted and free nitrogen is determined. With the Dumas method, gas volume is determined, corrected to standard conditions, and converted to mass equivalent. More recent instrumental methods have utilized conversion of nitrogen to an activated form and determination by chemiluminescence (6) and measurement by a flow injection technique (7). These procedures show

high correlation with values determined by the Kjeldahl method because all measure nitrogen present in the sample.

(b) *Peptide linkages.*—Because proteins are linear polymers of amino acids linked together mainly by peptide bonds plus some other chemical bonds, measurement of peptide linkages provides an indication of protein content. The principal methods are ultraviolet or near infrared absorption and complexation with copper ions. In the AOAC biuret method, **7.010**, for animal feed (5), a stabilized alkaline copper reagent is used to form a complex with 2 or more peptide bonds. The color of the copper complex as determined spectrophotometrically indicates the content of protein peptide linkages. The biuret test is commonly used in biochemical measurements for soluble proteins, but the test is not very sensitive, and glycerol, ammonium salts, sugars, and Tris buffer interfere in the analysis. Insolubility of meat samples precludes use of the biuret method. A pulsed nuclear magnetic resonance system (8) has been proposed for measuring the complexed copper in insoluble matrixes. Absorption of ultraviolet light by the peptide amide bonds has also been used in biochemical measurements particularly in liquid chromatographic applications, specifically by measuring absorption at 205, 220, and 235 nm. These wavelengths, however, are susceptible to interference from light scattering and by absorption by nonprotein components. Absorption by the amide linkages in the near infrared region apparently requires that the particles be of identical size for reflectance techniques (9).

(c) *Primary amino groups.*—Because proteins contain primary amino groups, their determination has also been used for protein determinations. In the tenth edition of *Official Methods of Analysis* (10), 2 methods were listed as official first action under Meat Products, but they are not included in the present edition. Method **23.017–23.018**, the Van Slyke nitrogen method, measured the nitrogen gas released by reaction of nitrous acid with primary amino groups; **23.019**, the Sorensen formol titration method, measured the ionization of primary amino groups by titration in the absence and presence of formaldehyde, an amino complexation agent. The principal procedure for determination of primary amino groups is the standard amino acid analysis which determines the content after hydrolysis by reaction with ninhydrin to produce a chromogenic substance proportional to amino acid content (11). Alternative reactants for quantitation of primary amino groups include fluorodinitrobenzene, fluorescamine, and phenylisothiocyanate (12). A method proposed by Horstmann in 1978 (13) uses the reaction of ninhydrin with the unseparated hydrolysate for estimation of total amino acids and therefore total protein. In this method, the relative content of amino acids in each protein must be determined first, because each protein has a different weight conversion factor and the contribution of imino acids must be known and a correction made. Although the factors for proteins range from 0.083 for elastin to 0.147 for protamines, most proteins apparently have factors between 0.11 and 0.12. A similar method for meat hydrolysates using trinitrobenzene sulfonic acid was proposed by Arneth in 1983 (14) for use in an automated system along with determination of the specific amino acid hydroxyproline.

Although all proteins contain nitrogen, peptide linkages, and primary groups, they differ in their content of specific amino acids. Chemical reactivities of various individual amino acid side groups can be determined on intact proteins or following acid, base, or enzymatic hydrolyses. Conversion of the content of specific amino acids to total protein assumes that the content of that amino acid is constant, and that no alterations occurred during preparation. With the commonly used acid hydrolysis procedure (11), for example, destruction of tryptophan, sulfur amino acids, and amides may occur.

(d) *Aromatic amino acids.*—The aromatic amino acids are phenylalanine, tyrosine, and tryptophan. The more common methods measure the phenolic ring of tyrosine; these include measuring absorption at 280 nm, and use of Folin's reagent, the Lowry procedure, Millon's reagent, and the xanthoproteic test (12). Ultraviolet absorption at 280 nm also measures contributions from phenylalanine, tyrosine, and tryptophan as well as nucleic acid components. The Lowry method is widely used in biochemical analyses for low levels of soluble proteins. It requires only about 5–10 μg/mL, but Tris buffer, EDTA, thiol reagents, sucrose, oxidized lipids, sulfhydryls, purines, hexosamines, amine buffers, phenol, magnesium, and potassium interfere in the analysis. The mechanism is not completely known but appears in part to involve complexation of copper ions in alkaline solution with peptide linkages (biuret reaction), followed by univalent reduction of the copper, and reaction of this copper with the Folin–Ciocalteau reagent to produce the blue complex. Aromatic amino acids may also be involved in the oxidation-reduction reactions, because proteins low in aromatics often have lower apparent protein values. A recent commercial protein determination uses a procedure similar to that of Lowry, except bicinchoninic acid replaces the Folin reagent and interference from the above substances is eliminated (15). Millon's reagent is used in method **7.011**, and the xanthoproteic test is method **7.014** (5). The Hopkins–Cole method and the Adamkiewicz variation for tryptophan determinations are methods **7.012** and **7.013**, respectively (5). These 4 tests are AOAC surplus methods.

(e) *Basic amino acids.*—Basic amino acids in acid medium are positively charged and can bind certain dyes, providing an indication of protein content. The proteins in meat have differing binding parameters. Seperich and Price (16) found that the sarcoplasmic proteins bind 27.0 ± 2.1 ng Acid Orange 12 dye/mg protein, the myofibrillar proteins 36.0 ± 2.8 ng, and the stromal proteins including collagen only 14.4 ± 2.0 ng. Histones, because of their high content of basic amino acids, would be expected to bind much higher amounts but these would only present a problem with organ meats. Consequently, dye binding procedures for meat protein estimation yield inaccurately low values with meats that have a high content of connective tissues or collagen. Other procedures for basic amino acids involve titration curves, the Sakaguchi method for arginine, and the trinitrobenzenesulfonic acid method for the epsilon amino group of lysine (17).

(f) *Imino acids.*—The imino acids, proline and hydroxyproline, are important in the connective tissue proteins collagen and elastin. Proline is a component of most proteins, but is present in high levels in collagen (about 11–14%) along with about 35% glycine and 11% alanine (12, 18). Hydroxyproline is also present in collagen in high levels (11–14%) and in low levels in some plant cell walls (19) but is not present in the skeletal muscle proteins. Consequently, determination of hydroxyproline content has been used as an indication of the connective tissue content (principally collagens). In the Woessner method or its variations, protein is hydrolyzed completely or partially to free amino acids. The hydroxyproline residues are oxidized with Chloramine T or periodic acid and are coupled with dimethylaminobenzaldehyde (Erlich's reagent) to give a chromogen that is measured spectrophotometrically (20). The content of hydroxy-

proline is multiplied by a factor to calculate apparent connective tissue. If a 12% hydroxyproline level is assumed, the factor is 8 (21); if the level is assumed to be 14%, the factor is 7.14. Determination of the connective tissue content of meats has been studied in Europe where methods are being considered for analytical determination of the fat-free, connective tissue-free meat. In an interlaboratory study reported by Dransfield et al. (22), determination of hydroxyproline content was the least precise procedure examined, with values ranging from 0.7 to 1.5% connective tissue content on the same sample and with 95% of replicates differing by up to 0.2%, well above the 0.06% suggested value. The calculated mean determinations of nonfat, nonconnective tissue lean meat from the various laboratories ranged 1.3%, from 19.9 to 21.2%. Correlation for glycine : hydroxyproline in collagen is 0.918, and for proline : hydroxyproline 0.80. Isatin and Sirius Red are histochemical dyes that react with proline and have been used for quantitation of connective tissues (23).

(g) *Sulfur amino acids.*—The sulfur amino acids are cysteine, cystine, methionine, cystathionine, and taurine. Only the first 3 are found in most meat proteins. The principal method involves use of Ellman's reagent which reacts quantitatively by a disulfide exchange reaction with the free sulfhydryl of cysteine (24). Some cysteine is present in nonproteins such as glutathione, and the reagent is also subject to interference by thionucleophiles (25). Processing often oxidizes some of the free sulfhydryls, altering their apparent content. The level of sulfhydryls may also be measured by the nitroprusside reaction or by titration with iodoacetate.

(h) *Amide content.*—Alkaline distillation has been used to determine the amide content of proteins (26), although near infrared spectroscopy measures some of the amide bonds along with the peptide linkages. Release of ammonia from the amide linkage during alkaline hydrolysis is rapid. Asparagine is a somewhat stronger electrolyte than is glutamine, more susceptible to nucleophilic substitution, and more likely to lose an amide proton. Extended time of distillation can cause release of ammonia from arginine (about 2% of total content) and from rearrangement reactions of serine or threonine. The amide content of proteins ranges from 2% for silk fibroin and 4% for collagens to about 26% for wheat gliadin, with most proteins about 8–9% (26). The alkaline distillation procedure has been proposed as a rapid method (less than 30 min) for estimation of meat proteins (27, 28).

(i) *Carbohydrate content.*—Many proteins are actually glycoproteins and contain carbohydrate residues. Most membranes contain gangliosides, complexes of carbohydrates attached to lipids, often combined with proteins. The quantitation of these residues as a function of protein level has not been used to any great extent except for soy proteins (29). With this method, the sample protein is hydrolyzed with dilute acid, the hydrolysate is neutralized, and the sugar content is determined by specific enzymes. Galactose is present in collagens, milk protein, and soy protein, whereas mannose is the principal carbohydrate in gliadin, a wheat protein.

(j) *"Crude" dry protein.*—Crude protein content is often approximated indirectly by calculating the weight remaining after removal of moisture and fat. This can be accomplished by rendering with heat or microwave energy or by extraction with specific solvents, followed by drying. With fresh meats, the correlation with protein content is high because of the lean-to-fat compositional relationship, but correlation is poor with processed meats where additives such as salt contribute to the total weight, giving false results.

Nonprotein Nitrogenous Compounds in Meat Products

Muscle proteins contain various nonprotein components whose content has been proposed as an indicator of muscle protein content. Creatine and creatinine are both present in fresh striated muscle, particularly the white fibers, and can be readily measured. Dvorak (21) reported that creatine values were not linear for total proteins (N × 6.25) but were linear for net protein values (total protein − hydroxyproline content × 8). Normal creatine levels are 23 mg/g net muscle protein, and creatinine levels are 1 mg/g. The nitrogen from these 2 substances would give an apparent 48 mg protein/g net muscle protein. During storage, creatine slowly dehydrates to form creatinine (30).

The methyl histidines also have been proposed for meat protein estimations (31). Methyl histidines are present in both the actin and myosin myofibrillar proteins and in the sarcoplasmic dipeptide buffers anserine and balenine. These beta-alanyl histidines can be extracted readily in fresh meats and determined by reaction with *o*-phthalaldehyde. Their levels show species differences. If the protein is hydrolyzed, the methyl histidines can be separated on ion exchange columns. The nonfat, nonconnective tissue free protein contains 6 mg 3-methyl histidine/g protein nitrogen.

Nonprotein extracts have been prepared with specific denaturants such as trichloroacetic acid, phosphotungstic acid, or barium hydroxide–sulfuric acid. Studies have indicated that not all the proteins are removed by such treatments. With 5% trichloroacetic acid, 21% of the actomyosin and 4% of the hemoglobin remained in solution (32). Phosphotungstic acid and barium hydroxide procedures appeared to be more effective but disposal was considered hazardous. In this safety aspect, it has been proposed that pure potassium sulfate plus hydrogen peroxide in the Kjeldahl procedure provides the same results at high digestion temperatures as does the potassium sulfate-heavy metal catalyst (33). Elimination of this use of mercuric oxide, copper, or selenium is beneficial in regard to exposure of the analyst and disposal to the environment.

Nitrogen-Protein Conversion Factors

The Kjeldahl method incorporates the assumption that all meat proteins have a mean nitrogen content of 16% with the mixture of amino acids. Calculation of the nitrogen percentage of the amino acid residues indicates that 13 have percentages less than 16 (Tyr 8.6; Phe 9.5; Met 10.7; Glu 10.9; Leu, Ile, and Hypro 12.4; Asp 12.7; Cys 13.6; Thr 13.9; Val 14.1; Pro 14.4; Try 15.0). Nine amino acid residues have nitrogen percentages greater than 16 (Ser 16.1; Hylys 19.4; Ala 19.7; Lys and Gln 21.9; Gly 24.5; Asn 24.6; His 30.6; Arg 35.9). Ammonia has a nitrogen content of 58.3%. The aromatic amino acids (phenylalanine, tyrosine, and tryptophan), the sulfur amino acids (cysteine and methionine), the acidic amino acids (glutamic and aspartic), and the imino acids (proline and hydroxyproline) all have nitrogen content below 16%, whereas the basic amino acids (lysine, hydroxylysine, histidine, and arginine), the simple aliphatics (glycine and alanine), and the amides (glutamine and asparagine) have higher contents. Free ammonia may be present from bacterial deaminations or from breakdown of amide linkages.

From published amino acid analyses (26, 34, 35), the weight percent content of the aromatic, sulfur-containing, basic, and amide amino acids in various meat components and products can be calculated as well as the nitrogen conversion factor (Table 2). Accurate amino acid determinations for certain proteins are difficult to locate in the literature, particularly

Table 2. Content of amino acid types and nitrogen-protein conversion factor calculated from amino acid data for muscle proteins and meat products[a,b]

Sample	Aromatic	Sulfur	Basic	Amide	Conv. factor
Collagen	2.2–3.6	0.5–1.1	12.8–14.9	0.2–2.5	5.24–5.48
Myosin	6.1–7.6	4.2–5.7	13.2–19.2	4.2–4.6	6.0–6.4
Actin	11.3	5.1	15.1	3.4	6.20
Albumin	12.7	7.6	22.0	3.1	6.28
Myoglobin	7.3	1.7	25.9	1.8	5.76
Lean meat	10.2	3.5	19.3	4.9	5.92
Mixed tissue	9.1	2.7	17.6	4.6	5.76
Adipose tissue	7.7	1.8	15.2	7.1	5.75
Connective tissue	8.0	2.6	14.9	5.6	5.66
Frankfurter	7.7	3.4	16.6	5.7	5.80

[a] Refs 26 and 34 were used for calculation of collagen, myosin, actin, albumin, and myoglobin; ref. 35 was used for collagen and the meat products.

[b] Values are expressed in sum of the relevant amino acids (g/100 g).

those with separate amide and tryptophan determinations. With muscle components the range of conversion factors is wide; for actin and myosin the factor is close to 6.25, whereas for collagen it is much lower. With meat products, the calculated factors were all below 6.0. A recent report by Yamaguchi (36) indicated that 5.73 was a better value for meat protein conversion, with factors for fruits, vegetables, and soybeans even lower.

With meat proteins, this lowered value arises from the presence of variable amounts of endogenous and exogenous nonprotein nitrogenous components and of connective tissue proteins, i.e., collagen and elastin. The correct factor for conversion appears to be directly proportional to a separate quantitation of these nitrogenous components and collagen. This would apply particularly to processed meats, because there are no minimum protein standards for fresh meats. Processed meats, however, can contain various regulated additives (1), some of which (soy protein, gelatin, and milk protein) do contain nitrogen. Certain products, e.g., head cheese or chicken roll, contain added skin which is high in collagen.

Determination of collagen content by determination of hydroxyproline levels and conversion can be done to estimate connective tissue content. In these cases the correct factor must be used. If the factor for collagen nitrogen were 5.7 rather than 6.25, there would be an 8.8% difference in calculated protein. If the factor is actually 5.4, the difference is 13.6%. This difference then directly affects the accuracy in determination of protein in certain meat products. Recent regulations (1) on defining the minimum protein fat-free (PFF) values for certain processed pork products are intended to indicate the apparent amount of water added during processing. PFF values are calculated by dividing the percentage protein determined using a total nitrogen method by a value of (100 minus the fat percentage). A cooked ham with a calculated crude protein content of 18% and a fat content of 10% would have a PFF value of 18/90 or 20.0. This would fall below the required PFF standard of 20.5 for common and usual hams. The regulations provide for subtraction of nonmeat proteins and gelatin that were added during processing, which necessitates additional analyses. Ham that contains excessive levels of connective tissue will indicate unrealistically high crude protein content.

Recent studies in our laboratory have indicated that the ratio of amide nitrogen (determined by direct alkaline distillation under defined conditions) to total nitrogen (determined by Kjeldahl) reflects the collagen content of the meat, because collagens have a low amide content. Other experiments on processed meats have indicated that an extraction with hot distilled water and characterization of the extracts for nitrogen can aid in detection of excessive additives and detection of nitrogenous constituents of lesser nutritional value than the essential amino acids.

For most accurate meat protein analyses, a combination of Kjeldahl with one or more parallel determinations is recommended. With fresh meats, separate determination of collagen content is recommended to correct the nitrogen-protein conversion factor. If the factor 5.40 for pure collagen is used, then the correct factor for collagenous meats is $(6.25 - 0.0085A)$, where A is percent collagen in the total protein. If the hydroxyproline-collagen conversion factor of 8 is used, the correct nitrogen-protein factor is $(6.25 - 0.068B)$, where B is percent hydroxyproline. By this method, a meat product containing 50% collagen would use the nitrogen-protein factor 5.825 rather than 6.25. This 6.8% difference could affect the values determined for PFF calculations. The choice of the additional method(s) is based on the user's requirement for protein characterization, available time, type of meat product, and sample size.

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Determination of Total Lipid and Lipid Subclasses in Meat and Meat Products

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Current interest in physiological and nutritional activities of the sterol, polyunsaturated fatty acid, and polar lipid fractions of meats and other foods indicates that analytical methods for lipids should be evaluated on their ability to recover and quantitate these classes. Current methods of lipid isolation furnish an extract that is dependent on the solvent(s) used, the type of food material, the temperature of extraction, and the relative proportions of the lipid classes present. Extraction with ethers or other relatively nonpolar solvents removes principally the neutral fats and nonpolar lipids. For an approximation of the crude fat content, such extraction is often sufficient, because the nonpolar fraction generally constitutes over 90% of the total lipids present. The polar lipids include the biochemically important (ω-3) and (ω-6) polyunsaturated fatty acid classes; thus, the method of lipid extraction of food products becomes relevant for a more complete and valuable characterization of their nutritional value. The various methods of lipid determination for meat products are examined for their total recovery of these important lipid groups. A sequential extraction in conjunction with subsequent analytical methods is recommended.

Today, interest in dietary fat is widespread because of either its beneficial or its purportedly harmful effects. This interest suggests a need for more complete labeling of food products for fat content—both *total* fat or lipid content and lipid composition. However, this need is not easily addressed, in part, because official methods used for lipid (fat) analysis only approximate the *total* lipid in meat and meat products, and methods that do report total lipid have not, for various reasons, been adopted as official methods.

To date, no exact definition of the composition of a lipid isolate has been agreed upon. The term lipid has been used traditionally to describe a variety of products that have in common solubility in solvents such as diethyl ether, hexane, chloroform, and/or methanol. A simplified classification of a lipid mixture isolated from muscle tissue covers most classes of compounds accepted as lipids. In this classification, lipids are grouped in 2 broad subclasses: the "simple" or "neutral" lipid class, which includes cholesterol (free and esterified), free fatty acids, and glycerides (mono-, di-, and tri-); and the second, the "complex" or "polar" class, which includes phospholipids and sphingolipids. The simple or neutral fraction also includes minor components, such as wax esters and hydrocarbons. The complex or polar lipid fraction contains most of those compounds (with the exception of cholesterol) that are of increasing interest to health professionals and consumers, e.g., the phospholipids, which contain high levels of the ω-3 and ω-6 fatty acids.

The terms neutral and polar have been used for some time to describe glycerides and phospholipids, respectively; however, *simple* and *complex* have been recommended to describe these subclasses because these terms are thought to have more precise meanings (1). The latter category was derived from the number of hydrolysis products that lipids form. Hydrolysis of a simple lipid such as a triglyceride yields 2 products/mol (glycerol and fatty acids), whereas a complex lipid such as a glycerol phospholipid yields 3 or more types of products (glycerol, fatty acids, phosphate, and an organic base).

Lipid Methodology

When AOAC methods are used for fat analysis, an extract is obtained which is termed "crude" fat (2). This term, although widely used, is not included in the simplified classification described earlier because it is not a clearly defined portion of the fat in meat and meat products, but represents instead only a portion of the *total* lipid recovered by extraction with either ethyl or petroleum ether, the 2 solvents specified for extraction. AOAC method **24.005**, a Soxhlet extraction technique, requires use of ethyl ether, although petroleum ether is sometimes used (2). Fat extractions by this method using the traditional apparatus require about 4–8 h for completion. An alternative method (**24.006–24.008**) uses the Foss-let apparatus, with tetrachlorethane as the extracting solvent (2). This extraction requires only about 30 min. However, although this method is precise compared with the Soxhlet method, it too yields a crude fat extract (3).

An earlier study by Hagan et al. (4) illustrates some of the problems associated with fat extracts obtained with crude lipid methods. Table 1 shows their results for extraction of fat from 3 cuts of beef, which were obtained by varying the extraction and drying methods and the extracting solvents. From these studies, it was established that the results obtained by the AOAC Soxhlet method are dependent on the solvent, the drying method, and the fat content of the sample. The Soxhlet method was also compared in this study with results obtained when the same samples were analyzed by the Bligh and Dyer method (5). The latter method uses a solvent combination of chloroform and methanol and has been used traditionally by analysts who require an intact *total*

Accepted March 24, 1986.
Presented at the Symposium on Critical Analysis of Analytical Methods for Meat Foods, 99th AOAC Annual International Meeting, Oct. 27–31, 1985, Washington, DC.

Table 1. Lipid content of beef extracted by several solvent systems and phospholipid content of extracted lipids[a]

Extn method	Solvent	Drying method	Total lipids extracted, %			Ratio, % PL/PL + TG[b]		
			Cut I	Cut II	Cut III	Cut I	Cut II	Cut III
Soxhlet	ethyl ether	freeze dryer	3.98	7.42	1.99	3.0	2.9	6.7
		vacuum oven	4.08	7.01	1.86	4.2	3.6	8.8
		air oven	3.98	7.03	1.82	3.8	3.9	9.6
	petroleum ether	freeze dryer	3.94	7.32	1.83	2.9	3.1	6.7
		vacuum oven	3.97	7.48	1.74	4.5	3.8	7.5
		air oven	4.00	7.56	1.74	3.7	3.2	8.0
	mixed ethers	freeze dryer	3.99	7.28	1.90	3.8	2.8	6.8
		vacuum oven	4.00	7.24	1.74	3.9	4.4	8.6
		air oven	3.94	7.40	1.84	4.1	4.0	7.7
	chloroform	freeze dryer	4.44	8.12	2.35	8.9	7.4	17.8
		vacuum oven	4.37	8.03	2.08	7.2	6.4	15.3
		air oven	4.44	8.25	2.04	7.4	6.9	14.0
Acid hydrolysis-Rohrig	ethyl and petroleum ethers	none	3.81	5.58	1.65	2.8	1.8	6.3
Bligh and Dyer	chloroform–methanol	none	4.60	7.17	2.50	10.1	8.3	18.3

[a] Adapted from ref. 4.
[b] PL = phospholipids; TG = triglycerides.

lipid extract that has not been altered by the extraction process. The chloroform/methanol method, which does not require drying the sample prior to extraction, invariably yields larger recoveries of lipids than does the AOAC Soxhlet method. This difference results in part from a more complete extraction of phospholipids by the Bligh and Dyer method. Table 1 also shows phospholipid recovery for the same samples.

These data indicate that the Soxhlet method gives varying recoveries of phospholipids, depending on the extracting solvent used, but regardless of the solvent, Soxhlet results are quite low compared with those obtained by the Bligh and Dyer method (5).

Methods such as the Soxhlet technique, which isolate a crude fat extract, are used primarily when a value for percent fat content is required for reporting purposes. However, today's consumers, nutritionists, and other health professionals would prefer methods that report total rather than crude fat values. Such methods are particularly needed when the amounts of cholesterol, polyunsaturated fatty acids, and phospholipids in animal-based products are required. The 2 methods traditionally used for determining total lipid were those developed by Bligh and Dyer (5) and Folch et al. (6). Both methods involve homogenization of the tissue, extraction of the resultant mixture in chloroform–methanol solvent systems, filtrations to remove residues, extraction or chromatography to remove nonlipid artifacts, and finally column chromatography to separate lipid classes (1). Although these methods are accurate, they require skilled operators and are time-consuming.

In recent years, our laboratory has developed a new method for lipid extraction, which differs significantly from other techniques and which obviates many of the problems encountered with traditional methodology (7, 8). This "dry column" method is quite versatile in that lipid may be isolated as 3 distinct fractions by simple changes in the solvents used.

Table 2. Comparison of lipid extraction methods[a]

Tissue	Dry column (sequential)			Folch
	Neutral fraction, %	Polar fraction (phosphatide), %	Sum total, %	Total (phosphatide), %
Lean pork	4.97	0.82 (0.64)	5.79	5.77 (0.67)
Fatty beef	29.35	0.67 (0.46)	30.02	29.87 (0.50)
Med. beef	10.08	0.72 (0.56)	10.80	10.70 (0.60)
Lean beef	3.43	0.80 (0.69)	4.23	4.38 (0.67)

[a] Adapted from ref. 8 with permission of the American Oil Chemists' Society.

A meat sample is placed in a mortar together with an antioxidant, ground with anhydrous sodium sulfate, and then blended with Celite 545. The powdered mixture is transferred to a glass chromatographic column containing a trap material of calcium hydrogen phosphate–Celite 545 (1 + 9). After the column is packed the lipid may be recovered in one of 3 ways: (*1*) Elution of the column with dichloromethane alone results in recovery of the "crude" lipid fraction (7). (*2*) Elution of the column with dichloromethane–methanol (9 + 1) results in recovery of a total lipid extract (7, 8). (*3*) Sequential extraction of the column first with dichloromethane to elute a neutral lipic fraction, and then with dichloromethane–methanol (9 + 1) to elute the polar lipid fraction (8). Lipid thus may be selectively isolated depending on subsequent analytical needs.

In our initial studies, the dry column method was compared with AOAC method **24.005** (2); nearly identical results for crude lipid could be obtained by either method (7). Later comparisons were made with the Folch chloroform–methanol method (8) where muscle tissue of varying fat content (3.4–29.3%) was analyzed by both methods for total lipid (as a sequential extraction) and phospholipid content (Table 2). These results indicate that sequential elution by the dry column method, which gives separate neutral and polar lipid fractions, results in a sum for these 2 fractions equal in amount to that obtained by the Folch method. Moreover, identical results were obtained for the phosphatides in these samples by both methods, and comparisons by thin layer chromatography gave additional verification for the equivalency of results between methods (8). A practical application for sequential separation of lipids in meat analysis is gas chromatography (GC) when a fatty acid profile of the lipids is required. Meat lipids typically are derivatized to methyl esters from a total lipid extract, and are subsequently separated on a GC system. By use of dry column sequential elution, the neutral and polar lipid fractions may be separated individually (9), which allows the analyst to examine the acyl composition of the phospholipid fraction more precisely. This

Table 3. Comparison of AOAC Soxhlet (7.056) and dry column methods for extraction of crude and total fat and phosphatide from canned pet foods[a]

	Dry column		AOAC Soxhlet	
	Total fat, %	Phospha-tide, %	Crude fat, %	Phospha-tide, %
Dog food				
Beef	9.60	0.76	8.65	0.48
Chicken	7.80	0.25	7.03	0.05
Lamb	5.18	0.73	4.50	0.32
Liver	6.41	0.99	5.63	0.48
Cat food				
Beef & liver	10.83	1.37	10.05	0.50
Salmon	9.08	1.43	7.58	0.46
Tuna	2.93	0.89	1.99	0.13

[a] Adapted from ref. 12.

Table 4. Comparisons of dry column and Roese-Gottlieb (Mojonnier) methods for lipid extraction from milk[a,b]

	Dry column		Roese-Gottlieb	
	% Total lipid	% Phos-phatide	% Total lipid	% Phos-phatide
Heavy cream	36.40	0.200	35.81	0.165
Light cream	17.53	0.119	16.87	0.087
Whole raw	4.41	0.039	4.02	0.030
Buttermilk	1.89	0.133	1.43	0.115
Skim	0.83	0.018	0.66	0.015

[a] Adapted from ref. 14 with permission of the American Dairy Science Association.

[b] All samples from same raw milk source.

feature is important because the polar lipids contain most of the biologically important polyunsaturated fatty acids. Such an approach was used in this laboratory to separate the lipids from hundreds of muscle samples in a study of dietary factors in beef animals (10).

Zubillaga and Maerker (11) modified the dry column method, which was originally developed for fresh meats, to analyze the lipids in nitrite-treated, heated meat and meat products. They found that subtle changes occurred in the resultant fractions when the sequential dry column procedure developed for fresh meats was used with cooked nitrite-treated meats. Neutral lipids recovered in the dichloromethane fraction were clear, but the polar fraction was bright yellow, a result of pigmented materials in the eluate. These pigmented materials catalyzed lipid oxidations and interfered with evaluation of possible antioxidant activity in the extracts (11). These authors corrected this problem by a slight alteration in the trap material of the chromatographic column, which allowed the polar fraction to be collected free of any pigmented contaminants. Other column modifications by these workers resulted in isolation of pigmented material in the polar fraction from cooked meat as a separate fraction, thus aiding its identification (11). Their efforts have assisted considerably in understanding the effects of oxidation on extracted lipids.

The dry column method has also been used for lipid isolation from foods other than raw and cooked meat and meat products. In a recent study (12), the method was compared with the AOAC crude fat method **7.061–7.062** (2) for canned pet foods. This study was instituted to provide an alternative to the official method where a nonflammable solvent extraction system could be used in place of the ethers needed with the Soxhlet apparatus. Results in Table 3 for various pet foods show that the major difference between the 2 methods is the amount of extractable phosphatide. The higher values for total lipid obtained by the column method result mainly from greater recoveries of phospholipid by this technique. Similar differences would be expected for other canned processed foods.

In addition to processed foods, applicability of the column method to other foodstuffs such as legumes (13) and milk products has been examined. Milk and milk products were extracted by the dry column method, and the results were compared with those obtained by the Roese-Gottlieb (Mojonnier) method for lipid extraction (14). In this study, milks of varying fat content were analyzed initially for total lipid and phospholipid by the 2 methods (Table 4). Slightly higher total lipid values were obtained by the dry column method than by the Roese-Gottlieb method. More important, phosphatides were recovered in higher yields by the former method. This finding is significant because milk contains small amounts of phospholipid and the Roese-Gottlieb method, which uses ammonium hydroxide in the extraction process, may cause alteration or destruction of certain sensitive polar lipids such as phosphatidyl serine (15).

The dry column method was recently applied by Hundrieser et al. (16) to isolate lipids from human milk. This work was undertaken to find an alternative to the Folch or Roese-Gottlieb method. Agreement between the Folch and dry column methods was good. Use of the latter method also avoided the difficulty of time-consuming separations in the Folch method where recalcitrant emulsions are often formed during extraction.

The dry column method also has been modified by other researchers to determine nitrosamines in bacon and other cured meat products (17). The method used by the Food Safety and Inspection Service (FSIS) to determine violative levels of nitrosamines was the lengthy mineral oil distillation–gas chromatographic–thermal energy analyzer screening method (17), which limited analyses to about 6 samples/day/analyst. In comparison studies, the modified dry column method was rapid (20 samples/day/analyst), less susceptible to artifactual nitrosamine formation, and equally as precise as the FSIS method (17). The column procedure was collaboratively studied (18), and adopted by AOAC (19).

Numerous methods are available to the analyst for isolating lipid from meat and meat products. The choice of method, however, will influence the composition of the resultant lipid extract and its suitability for further analytical studies. Although method choice presently may be governed by official reporting requirements, concerns now expressed by consumers and health professionals regarding the completeness of published values for lipid composition of foods may result in efforts to substitute total rather than crude fat methods for such purposes and may lead to a reassessment of the methods currently used for fat analysis.

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Chemical Analysis of Meat Products

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Meat, particularly muscle tissue, is basically a 3-component system of protein, moisture, and fat. Although this seems a simple analytical system in which to monitor product composition for regulatory compliance, the simplicity quickly erodes when meat is formulated into the broad variety of products commercially available today. Alternative protein sources, as well as preservatives, binders, extenders, emulsifiers, spices, and other flavoring ingredients, add to the analytes of concern and highlight the need for analytical methods suitable to support inspection and labeling requirements to ensure product compliance. Some key issues are noted which involve protein quality analysis, rapid compositional analysis, isolated soy protein analysis, and minced fish in meat products.

Some analytical issues that typify the kinds of regulatory concerns to be addressed in the chemical analysis of meat and poultry products are discussed in other papers in this symposium (1–4). These issues include recent efforts to determine protein quality to support regulatory initiatives on mechanically separated meat and poultry; progress on a rapid immunological screening method for isolated soy protein, which would support in-plant inspection; the impact of rapid developments in food technology, exemplified by use of marine fish products as a protein supplement in meat products to produce a vast array of new food commodities; and an economic-regulatory issue of identifying accurate, more cost-effective methods for analysis of meat food products to ensure, among other things, compliance with product standards, labeling or monitoring, and the effectiveness of approved quality control programs. Some background information will provide depth and meaning to the Food Safety and Inspection Service (FSIS) program responsibilities.

Meat Inspection

In the United States in 1984, 127.7 million red meat animals were inspected for slaughter. This number included approximately 38.3 million cattle and calves, 82.7 million swine, and 6.4 million sheep. In the poultry industry, 4.56 billion birds were inspected for slaughter, of which 4.38 billion were chickens. Inspectors examined an estimated 55 billion pounds of meat and poultry and 119 billion pounds of processed products, a 29% increase in the last 5 years. Meat and meat product imports were slightly in excess of 2 billion pounds from approximately 1100 certified foreign establishments.

In our 3 field laboratories and their respective contract laboratories, over 240 000 analyses were performed, including 96 000 in food chemistry, 19 000 for food microbiology and species identification, 37 000 for chemical residues, 45 000 for antibiotic residues, 11 000 for pathology, 17 000 for serology, and 14 000 for food additives and nonfood analyses. Of these samples, approximately 113 000 were taken from processed products such as hams, sausages, and cured meats. The 37 000 chemical residue samples analyzed covered approximately 100 different analytes, typically at levels of interest of less than 1 ppm and, not too uncommonly, at the low parts per billion level. Some of those with a vested interest in FSIS operations wish we would analyze for a broader variety of analytes to protect the health of consumers more effectively.

Recent emphasis on veterinary drugs, certain pesticides, and anabolic drugs has required methods for screening, quantitation, or confirmation to low or sub parts per billion levels. These levels require meticulous, exacting sample analyte preparation on a relatively large number of samples per day. Furthermore, costs associated with collecting, preparing, and shipping requested samples for laboratory analysis are high. Thus, we need to develop rugged, reliable, and rapid screening procedures to provide more flexibility in our residue monitoring strategy and so that we do not use a disproportionate share of our laboratory analytical resources for those samples that have nondetectable levels of analytes.

To avoid giving an inappropriate perspective of the laboratory analyst's role, it should be noted that monitoring the performance of the meat and poultry industry and the respective products is primarily the responsibility of the inspectors in the producing establishments. If an inspector is concerned about the safety or quality of a product or if a program is designed to generate samples for chemical analysis, samples are taken by the inspector for the laboratory. The laboratory analysis, thus, is an adjunct of the inspection operations. The Compliance Division of USDA's Meat and Poultry Inspection Operations systematically monitors meat and poultry products, and its activities are closely coordinated with in-plant inspection.

Accepted March 24, 1986.
Presented at the Symposium on Critical Analysis of Analytical Methods for Meat Foods, 99th AOAC Annual International Meeting, Oct. 27–31, 1985, Washington, DC.

Technical Aspects of Meat

In simple terms, traditional meat is skeletal muscle derived from bovine, ovine, porcine, and poultry carcasses; this does not intend to exclude those items found in typical sausages or major organ meats such as liver, kidney, and tongue. Nontraditional animal protein sources would include "all other" protein sources. These descriptions may not be all encompassing, but will serve for this discussion.

Meat, particularly muscle tissue, is basically a 3-component system of protein, moisture, and fat. Although this seems a simple analytical system in which to monitor product composition for regulatory compliance, the simplicity quickly erodes when meat is formulated into the broad variety of products commercially available today. Many of these products result because of use of approved food additives. Although limited amounts of these additives are necessary to guarantee adequate food supplies for a growing population, their use is strictly controlled by laws that assure consumers that foods are safe to eat, wholesome, and accurately labeled.

Meat Additives

Under the Food, Drug and Cosmetic Act, the term "food additive" is defined as any substance which "results or may reasonably be expected to result—directly or indirectly—in its becoming a component or otherwise affecting the characteristics of any food." The definition includes any substance used in producing, processing, treating, packaging, transporting, or storing food, including any sources of radiation intended for such uses.

The 1958 Food Additives Amendment to the act exempted 2 groups of additives from the testing and approval process. The first is the list of substances which experts have classified "generally recognized as safe" (GRAS). This list includes substances such as flavorings and spices, which are considered harmless because past extensive use has produced no known harmful effects. Also exempted from testing are "prior sanctioned substances" that the Food and Drug Administration had approved for use in food prior to passage of the 1958 Food Additives Amendment. Additives can be removed from the lists, however, if tests indicate the substances are not safe for human consumption.

The 1960 Color Additives Amendments brought all colors—natural and synthetic—under the Food, Drug and Cosmetic Act. Color additives may not be used to deceive consumers or to conceal blemishes or inferiorities in food. Tests are available for the presence of synthetic dyes, such as FD&C Reds 1, 2, 3, 4, and 40; Yellows 1, 4, 5, and 6; Blues 1 and 2; Greens 2 and 3; Violet 1; Methyl Violet; Orange B; Oranges 1 and 2; and Rhodamine B. The Food Additives Amendment and the Color Additives Amendments include the so-called Delaney Clause, which prohibits the approval of an additive "if it is found to induce cancer when ingested by people or animals," or "if it is found, after tests which are appropriate for the evaluation of the safety of food additives, to induce cancer in people or animals." Any substance found to induce cancer would be regulated under the general safety provisions of these laws, as well as by the Delaney Clause.

Direct additives are substances added directly to foods for specific purpose, and, by law, they must be named on labels on meat and poultry products. Meat and poultry inspection regulations specify how these additives can be used.

Antioxidants, such as propyl gallate and butylated hydroxyanisole (BHA), are approved for use in retarding rancidity in dry sausage, rendered animal or vegetable fat, fresh pork sausage, and dried meats. Ascorbic acid and citric acid are antioxidants used in curing to accelerate color fixing or to preserve color during storage. Citric acid also helps protect flavor and increases the effectiveness of other antioxidants.

Preservatives and curing agents such as table salt, sugar, benzoic acid, and sodium nitrite help prevent food spoilage. The limited use of nitrite is permitted to cure products like bologna, frankfurters, bacon, and salami, and to prevent the growth of organisms that cause botulism in humans. Benzoic acid and its derivatives are not allowed in meat products.

Other additives are normally identified as binders. These are the cereal flours, and they can usually be determined quite easily. The use of cereals has decreased in recent years, because the original function of providing additional protein to increase meat emulsion stability is accomplished more effectively by the protein concentrates and isolates. Nonfat dry milk and soy protein products are permitted in such items as sausages and meat patties to bind ingredients and to extend processed products. A solution of potassium sorbate is permitted to coat sausage casings to retard growth of mold. Proteolytic enzymes, such as bromelin, ficin, and papain, are permitted for the purpose of tenderizing beef cuts.

Sugars as additives are self-limiting because of taste. However, corn syrup solids, because of their different degrees of hydrolysis, can be used as fillers. Analysis is possible for maltose, but the calculation converting maltose to corn syrup solids is not specific and may not be accurate. With the development of liquid chromatographic (LC) techniques, it may be possible to estimate the amount that has been added.

Another major group of additives to the basic 3-component meat system is the nonmeat proteins. Addition of nonmeat protein also provides the opportunity to add additional water and fat to provide a product that would have a similar "proximate" composition to an all-meat product. The development of additional "exogenous" protein isolates from other vegetable products such as cottonseed, peanut, pea, and alfalfa, in addition to the single-cell yeast proteins and the proteins from blood and bone, has created additional components, the regulation of which has yet to be resolved.

On occasion, we have been asked to analyze meat food products for chemicals that affect the color of heme pigments in meat. Compounds such as imidazole and sulfite have such an effect. Histamine and histidine are reported also to fit into this category.

Nonmeat Proteins

Proteins in foods have unique chemical and physical properties, including gel formation, viscosity, emulsification, water absorption, fat absorption, texture, flavor, and color. The protein additives *need* to perform the same functions in foods as the more expensive animal protein that is either augmented or replaced. Food technologists have been successfully exploring a wide variety of proteins and their properties for use in meat food products.

Use of nonmeat proteins in meat products has been growing rapidly because of economics and developments in food technology. Consider the following factors. The global rise in population is focusing attention on the need for protein-rich foods, particularly traditional animal products such as meat, milk, fish, eggs, and cereals. Limitations on land and protein feed resources require that they be used efficiently. Economic forces, the generally narrow profit margin in the industry, create shifts in supply and utilization of traditional meat protein sources. As a consequence, alternative protein sources are being developed that provide the same properties

at an equivalent nutritional level and lower cost than meat protein.

The economic basis of protein production is the cost of converting raw agricultural products to edible proteins. Substantial efforts and some noticeable gains have been made in the efficiency of converting feed grain protein to animal protein. However, much feed grain protein is lost by the time the edible protein from meat and poultry sources is available for human consumption. Thus, it is not too surprising to see the increased use of direct vegetable protein processing into food production. Protein food ingredients can supplement such products as sausages, canned meats, processed hams, and ground meat. The use of nutritional, agricultural food protein ingredients will allow more meat products to be made from the same supply of meat or the same quantity of meat products to be made from a lesser quantity of meat.

However, in countries such as the United States that have protein-rich staple foods derived from meat, poultry, milk, and dairy products, these other protein sources, the cereals, grains, and vegetable proteins, will probably have to acquire a share of the market as protein substitutes or extenders. This usually creates a basis for regulating their use. In the United States, several are regulated for typical uses. Dried skim milk, soy flour, soy protein concentrate, nonfat dry milk, and cereals are limited to 3.5% in meat products. Isolated soy protein is limited to 2%. Whey may be used at levels of 3.5% for most products, but 8% in some products, and "sufficient for purpose" in others. Sodium caseinate, hydrolyzed plant protein, milk protein hydrolysate, and vegetable starch may be used at levels "sufficient for purpose." Other vegetable products are a possibility for the future. Single cell protein sources, spices, and flavorings complement the list of potential nonmeat proteins of which we must be aware.

The use of other protein sources is not approved at the present time, although several requests or inquiries have recently been submitted for use of fish protein isolate, fish protein concentrate, protein extracted from pork bones, and pork skin. While plant proteins other than soy are not currently permitted for use in meat products, the situation may change in the future. Work is being reported in the literature for many plant proteins (wheat, cottonseed, etc.), and immunological methods are being developed (5). As with soy protein, there is an inherent need to obtain antisera to a specific protein component, which could be utilized in a method development.

The principal motivation to use these materials is, again, economic: nonmuscle proteins such as bone or skin protein, yeast, whey, soy products, nonfat dry milk, and cereals are less expensive than muscle protein. Many of these materials, particularly alternative animal protein substances, are difficult to identify by chemical testing, because muscle protein has a multicomposite amino acid composition.

A relatively new product finding application in the European meat industry, but currently not permitted in meat products in the United States, is bone protein. Little is known about the composition and protein character of the product. It is possible that such products might be produced domestically in the future. Bone protein was initially derived from pork bones. More recently, similar products from cattle and poultry are being mentioned. We are concerned that these protein sources may be low in essential amino acid content and elevated in cholesterol content. Thus, a research effort on safety seems warranted and is being considered. Whether a suitable method for quantitation of bone protein can be developed will await research results.

Other protein sources have our attention. A commercial product of edible protein made from fresh pork rinds is available, whose advertising includes advantages such as it being a natural meat product and that it may be analyzed as a part of meat content where it is legal to use. The product has a fairly low (14%) essential amino acid content and a high moisture protein equivalent of 5 to 7, thus, it could yield products with higher moisture content. We need to continue our search for acceptable methods of protein analysis that meet the criteria of scientific and regulatory acceptability; we must move quickly, because food technology is moving rapidly to find new ways of using protein products.

With this setting highlighting the need for many analytical methods to support inspection operations, let us consider some of these analytical problems.

Amino Acid Analysis

In his paper on amino acid analysis for meat protein evaluation, presented at this symposium, Ashworth (1) highlights our efforts in LC methodology for amino acid composition. Regulatory requirements for product quality are based, among other things, on the percentage of essential amino acids to total amino acids. A USDA-sponsored Expert Work Group proposed using an upper limit of hydroxyproline content of meat and poultry products as a regulatory tool (6). It has also been suggested that the amount of meat protein of a product be quantitated and the amount of nonmeat protein be determined by a difference method. Although a considerable list of components unique to meat can be identified and quantitated, variation due to differences in meat cuts and organs is not completely defined. Quantitation of meat protein in a product as a worthy approach to the problem is not fully resolved. Laboratories in Great Britain, The Netherlands, and Denmark are evaluating or considering evaluating quantitation of 3-methyl-histidine as a measure of meat protein (personal communication, Roger Wood, Ministry of Agriculture, Fisheries and Food, UK, 1984; Paul Beljaars, Food Inspection Service, The Netherlands, 1984). It may be a reasonable approach, particularly if it is used in conjunction with another analytical tool such as hydroxyproline for collagen content or multivariant amino acid regression analysis.

We have considered these alternatives in our approach. Ashworth (1) also describes results on the influence of carnosine, anserine, and balenine (all β-alanine derivatives) on the essential amino acid content of mechanically separated meat and poultry, and he addresses means of screening for hydroxyproline content.

Isolated Soy Protein

Protein derived from soy is used in at least 30 countries, and soy is the second largest agricultural crop in the United States. Its use as a protein source is substantial. The need for an acceptable, rugged, accurate method for isolated soy protein (ISP) has been an international concern for more than a decade; international organizations in Europe and North America have established working groups on the topic—with only limited success. In the United States, recent changes in the regulation for ISP, removing the titanium dioxide tagging agent, have left us without a sufficient analytical method to support inspection controls for this protein source. Nevertheless, we believe there are still avenues of approach that are technically feasible.

A regulatory procedure for soy protein must meet the following criteria: (*1*) differentiate soy proteins from other nonmeat proteins as well as meat proteins; (*2*) qualitatively and

quantitatively differentiate ISP, soy concentrate, and soy flour, and be applicable when any of the soy proteins is used; (3) be applicable irrespective of the process used to manufacture ISP, soy concentrate, or soy flour; (4) demonstrate reproducibility and repeatability within acceptable limits.

In this symposium, Berkowitz (2) describes immunological approaches to ISP analysis as well as how they fit into our strategy of developing procedures to fit the 4 possible combinations of ISP solubility (low and high) and regulations (approved and unapproved use).

Minced Fish in Meat Products

In the United States, minced fish has no market other than for use in pet foods because fish protein is not presently allowed in meat and poultry products. For more than 10 years, the fish industry and the U.S. Department of Commerce have been trying to get this changed. FSIS has been requested to make rules to permit the use of minced fish. Scientific work has shown that franks and other sausages containing 10–15% minced fish are acceptable; higher levels change the taste and are unpalatable (7).

Rasekh (3) reviews the history of minced fish in meat products and addresses such topics as the high economic value of fish; its high protein and generally low fat content; advances in automated deboning; types of products in which minced fish may be appropriate; processing economics and technology; nutrition quality; and hazard assessment, such as microbial contamination, enzymatic stability, pesticide residues, toxic substances, bone particles, and micro constituents.

Meat Protein Analysis

For assessment of protein content of meat products, we have relied primarily on the Kjeldahl method for measuring total nitrogen and then multiplying that result by an appropriate factor to give apparent protein. However, other determinations such as the biuret method for peptide bonds, the ninhydrin method for amino acid amine groups, the Lowry method for tyrosine content and some reducing compounds, the dye binding method for charged groups, and amino acid analysis are alternative ways of measuring protein which are worthy of consideration. Analytical procedures for water and fat are straightforward and are generally simple in theory. There are presently several AOAC official methods for each of these analytes, but they are not all equivalent for use in our laboratories. Some are too labor-intensive for the large numbers of samples we must analyze, some require calibrations on a product-by-product basis, and others have unacceptably small or large sample size requirements.

McNeal (4) reviews the 25 instruments and techniques evaluated before 1980 and the 38 instruments and techniques we have evaluated since 1980, and comments on whether they fit our program. Our concern is to meet regulatory requirements, which are generally more stringent than other needs. This does not necessarily imply that they are better, but that we at FSIS must meet different needs. In fact, many of the methods that do not fit our program may be suitable for in-plant quality control programs designed to provide rapid techniques for monitoring process control.

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Amino Acid Analysis for Meat Protein Evaluation

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The Food Safety and Inspection Service procedure for determination of essential amino acid content of mechanically processed products from red meat animals and poultry is based on hydrolysis of a powder prepared by blending samples in acetone-chloroform. The hydrolysis procedure incorporates thioglycolic acid to prevent loss of tryptophan. Aliquots of prepared hydrolysates are injected into a liquid chromatographic system, using gradient elution on an ion-exchange column for separation. The system also uses post-column hypochlorite oxidation coupled with orthophthalaldehyde reagent and fluorescence detection. Modification of the elution program allows concurrent determination of tryptophan with minimal added cost. Chromatograms from beef, pork, and poultry products show adequate separation and quantitation of β-alanine, 1-methyl-histidine, and 3-methyl-histidine, indicating that the procedure could be used to estimate muscle content of products. A colorimetric procedure for assay of hydroxyproline was introduced and validated as an adjunct method for protein quality estimation.

Accepted March 24, 1986.
Presented at the Symposium on Critical Analysis of Analytical Methods for Meat Foods, 99th AOAC Annual International Meeting, Oct. 27–31, 1985, Washington, DC.

Mechanically deboned or separated meat products have been produced for over 15 years. In the late 1960s, the Food Safety and Inspection Service (FSIS) and its predecessor organizations first formulated regulations to control the minimum quality requirements for mechanically separated products from red meat animals and poultry. Mechanical deboners were first used in poultry processing plants to more efficiently harvest the skeletal muscle on chicken parts of lesser economic value. Necks, backs, and frames processed through mechanical separators yield products that are generally of very acceptable quality. Many new products that utilize mechanically processed poultry have appeared on the market in the last 10 years; most have appeared during the past 4 years as newly formulated "fast food" products such as chicken nuggets, patties, steaks, and other poultry products which have gained high market acceptance.

Mechanically separated products are produced by grinding and crushing relatively soft bones, separating and excluding bone fragments by a series of screens or baffles, and extruding the final product through plates with very small diameter orifices or holes. Obviously, the skeletal muscle obtained is diluted by periosteal cartilage, connective tissue, fat, and bone marrow in the case of red meat animals, and in poultry or turkey products by extruded skin as well.

The yield of mechanically separated products from red meat animals such as veal calves, cattle, swine, and sheep is limited to those bones that can be readily crushed and ground. The majority of such red meat products therefore come from ribs, necks, and backbones, with lesser amounts from hams and shoulders in the case of pork, and very little from leg bones of mature beef animals.

Current Regulations

Current regulations (1) on mechanically separated products from red meat restrict bone to 0.85% maximum calcium content. Bone particles are limited to a maximum size of 0.85 mm in greatest dimension. Maximum allowance for fat is 30%, and 14% is the minimum acceptable protein content. Protein quality is controlled by requiring a protein efficiency ratio (PER) of at least 2.5 (equivalent to that of casein) or by an alternative measurement of the essential amino acid content of the protein. Current regulations specify a minimum essential amino acid content of 33%, calculated as the total of isoleucine, leucine, lysine, methionine, phenylalanine, threonine, and valine divided by the sum of alanine, arginine, aspartic acid, glutamic acid, glycine, histidine, hydroxyproline, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryosine, and valine. This value is expressed as g essential amino acids/100 g amino acids.

The way in which the regulation is stated is important because it allows the amino acid analysis to be performed and accurately calculated without a requirement for proximate analyses. Indeed, because mechanically processed products contain quantitites of nonprotein nitrogenous products such as purines, pyrimidines, free amino acids, dipeptides, and nucleic acids from bone marrow and muscle, estimation of protein by the Kjeldahl method does not provide a very accurate measure of protein content. In one attempt to measure total nitrogenous substances, Happich et al. (2) found that only about 85% of Kjeldahl nitrogen could be accounted for in products from red meat animals.

The current regulation requiring measurement of essential amino acids content does not require estimation of either trytophan or cystine. The requirements for these 2 amino acids were deleted because accurate measurement of either requires an extra sample analysis, extending analytical requirements, and because all commercial laboratories charge extra fees ranging from $25 to $50 for analysis of either cystine or tryptophan (FSIS survey, 1982).

Compliance

To demonstrate compliance, a producer must submit 3 amino acid analyses, spaced at 1 month intervals during a 3 month period, from 1 lb composite samples taken over the course of a 12 h production shift, then analyzed and submitted by the producer. During the testing period, an unstated number of samples may be taken by FSIS inspectors; all must be in compliance. Once compliance is demonstrated, sample analyses are required only once every 6 months. If any sample analysis fails to meet compliance, amino acid sampling for compliance verification is again required. Samples not meeting the protein quality requirement may be used only for fat rendering.

Other compliance measures require submission of 10 consecutive 1 lb samples from each production lot. Analyses for calcium, fat, and protein content are taken during this period. Samples failing to meet requirements for minimum protein content of 14%, or exceeding the maximum fat allowance of 30%, must be designated by the phrase "for processing" and may be used only to formulate products where a standard of identity sets the protein and fat requirements.

Products meeting compliance may be used in certain specified products to replace up to 20% of the muscle meat block content.

Analysis

The FSIS method for essential amino acids was developed in the laboratories of the Chemistry Division Laboratory Branch. The early version of the method developed for application to standard commercial amino acid analyzers used ninhydrin as the post-column detection system.

The more critical parts of the analysis are sample defatting and preparation and hydrolysis. Regulatory allowance of 30% fat requires that a defatting step be included in sample preparation. The original sample is reground and mixed, and a 2–3 g subsample is homogenized with 30 mL acetone–chloroform (3 + 1). The homogenate is poured into a coarse porosity glass fritted filter, and the homogenizer blade is rinsed and cleaned with a further 30 mL portion of the defatting solvent. The rinse is added to the filter, vacuum is applied, and the sample is air-dried. The dry, free-flowing powder produced is weighed into a special 2-piece tube for hydrolysis. The powder is stable and may be held for an indefinite period before analysis.

The hydrolysis procedure utilizes addition of thioglycolic acid (3) to the protein sample to avoid destruction of tryptophan during hydrolysis. Norleucine is added as an internal standard. After addition of hydrochloric acid, the prepared hydrolysates are evacuated to an internal pressure of 50 millitorr, and then hydrolyzed for either 24 h at 110°C or 4 h at 145°C. After hydrolysis, HCl is removed under vacuum and the dried residue is taken up in 5 mL of the appropriate buffer for analysis. A 20 μL sample is applied to the amino acid analyzer. The diluted hydrolysates may be refrigerated for several days if necessary.

Mechanically separated (species) products may contain considerable quantities of collagen. Therefore, hydroxyproline must be separated and quantitated. The original version of the FSIS essential amino acids analysis method (4) used a 4-buffer stepwise elution following the sequence pH 3.00, 3.25, 4.25, 7.90 for ion-exchange separation. An example of such a separation is shown in Figure 1. Sample load in this case is 10 nmole of each constituent amino acid.

LC adaptation of method.—Because our laboratories were spending considerable sums of money for amino acid analyzer maintenance and the manufacturer was phasing out the analyzers, we decided to modify the amino acid method and adapt it to liquid chromatography (LC) with postcolumn addition of orthophthalaldehyde (OPA) and fluorescence detection. Such a system had several advantages over the standard amino acid analyzer methodology: (*1*) sample injection conditions were simpler and more reliable; (*2*) LC equipment did not have to be totally dedicated to amino acid analysis; (*3*) the gradient ion-exchange separation required only 2 buffers that could be prepared in the laboratory, eliminating pur-

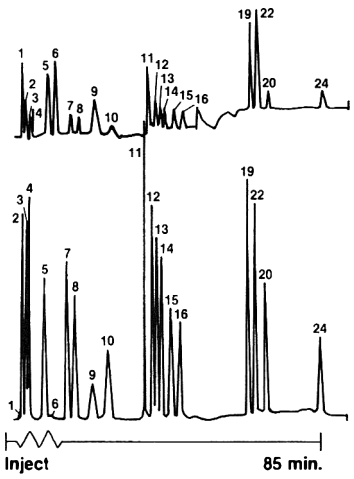

Figure 1. Separation of amino acids in mixed standard. Dionex amino acid analyzer, ion-exchange separation. 10 nmole column load. Detection: lower trace 570 nm; upper trace 440 nm. Peak identification: 1, Oh-Pro; 2, Asp; 3, Thr; 4, Ser; 5, Glu; 6, Pro; 7, Gly; 8, Ala; 9, Cys; 10, Val; 11, Met; 12, Ile; 13, Leu; 14, Norelu (internal standard); 15, Tyr; 16, Phe; 17, β-Ala; 18, 1-Mehis; 19, His; 20, NH_3; 21, 3-Mehis; 22, Lys; 23, Trp; 24, Arg.

chase of commercially prepared buffers; (*4*) postcolumn reagents were less expensive and were simple to prepare; (*5*) sensitivity of detection was greatly increased. Figure 2 shows a comparison of the instrumental layouts for amino acid analysis by both ninhydrin and postcolumn OPA detection.

The ion-exchange separation developed for the OPA method uses a 2-buffer gradient beginning at pH 2.95, 0.2M Na^+; column temperature is 52°C. The separation utilizes 2 sequential concave exponential gradients followed by a hold at 100% B buffer (pH 7.1 and 1.1M Na^+). This hold also effectively strips the column. After a re-equilibration period in which column conditions are returned to 100% A buffer, the next sample is injected. Total program time is approximately 120 min. Figure 3 shows a typical separation by this technique. Column load is 5 nmole. We used a Waters Associates LC system; gradient conditions and program times are shown in Table 1.

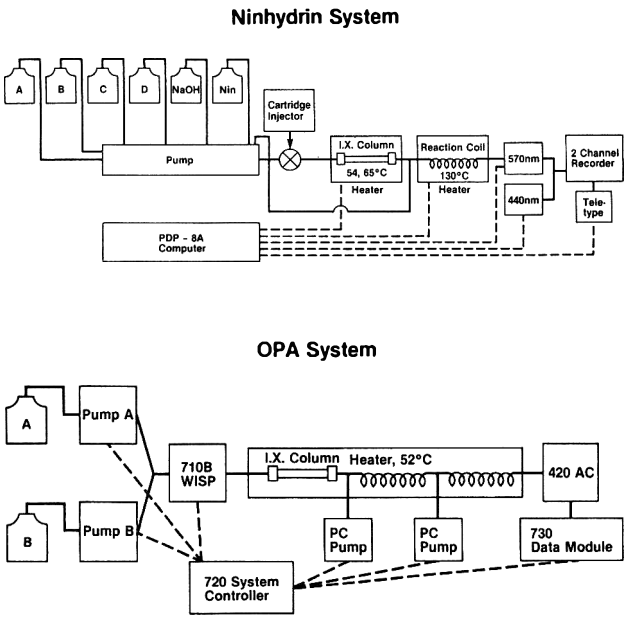

Figure 2. Instrumental layouts of ninhydrin and orthophthalaldehyde detection systems for amino acids.

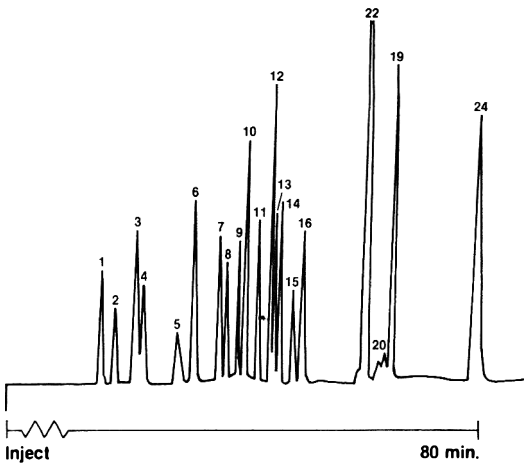

Figure 3. Amino acid standard separation by liquid chromatography. 5 nmole column load. See Figure 1 for peak identification.

Analytical Requirements

Rigid analyst quality assurance and analyst familiarization procedures must be required and maintained if the results of such a complex analysis are to remain credible. Analyst familiarization procedures in FSIS laboratories comprise 3 phases; the requirements are shown in Table 2.

Other requirements provide that no sample can be used for analytical reporting if recovery of the internal standard, norleucine, is less than 90%. All sample analyses are reported as the average of computed essential amino acid contents of 2 separate sample hydrolysates. This was deemed necessary because of inhomogeneity encountered in duplicate weighings of the sample powder. Essential amino acid contents for

Table 1

Gradient Conditions and Program Times

Pump	Solvent	Comp
A	Buf A	2.95
B	1.1 Na	7.10
C		0

Initial Conditions/Gradient Table

Time	Flow	%A	%B	%C	Curve
Initial	.50	100	0	0	•
45.00	.50	20	80	0	08
60.00	.50	0	100	0	08
80.00	.50	100	0	0	11
120.00	.20	50	50	0	11

External Events

No.	Description
1	Hypo
2	OPA

Time	No.	Status
10.00	1	On
10.10	2	On
85.00	1	Off
85.10	2	Off

Table 2

Amino Acid Analyst Familiarization Requirements

Phase 1: Set up gradient elution program and produce a series of ten consecutive standard runs. Calculate the coefficient of variance (COV) for each of the twenty (20) component amino acids* and ammonia present. The average of the sum of all amino acid COV's must be 3 percent or less to be acceptable.

Phase 2: A familiarization sample of mechanically processed beef is analyzed, producing a set of at least ten analyses. Each analysis value is the average of two separate hydrolysates, and the essential amino acid percentage is calculated on each hydrolysate. No sample whose internal standard recovery is below 90 percent is used for calculation; assays on such samples are repeated. Also, no sample duplicate averaging is allowed on samples if duplicate essential amino acid contents differ by more than 10 percent. The computed COV for this sample set must not exceed 5.0 percent.

Phase 3: Completion of the familiarization sample triggers the sending of a final check sample. The check sample is analyzed producing a set of ten analyses in which the COV of the essential amino acid content is 5.0 percent or less and the difference from the known value of the essential amino acid content is 5.0 percent or less. All of the requirements of phase 2 must be met as well.

*The amino acid standard mixture contains added hydroxyproline, norleucine and tryptophan.

each analysis are calculated but are averaged only if the calculated essential amino acid contents for the duplicates differ by less than 10%. Otherwise, analyses must be repeated.

Tryptophan

The LC method (5) for analysis of essential amino acids has been used for 2 years in the FSIS Midwestern Laboratory. Last year, because of an agency need for determination of tryptophan in mechanically separated poultry products, we were asked if the amino acid method could be modified to include tryptophan analysis without excessive added cost.

The original hydrolysis procedure was designed to protect against destruction of tryptophan. Experience in our laboratory indicates that it protects methionoine as well. Because of the need to monitor both proline and hydroxyproline, postcolumn hypochlorite oxidation was built into the procedure to provide ring cleavage of these 2 amino acids to produce OPA-reactive products. Although standard amino acid mixtures containing tryptophan do produce a peak at the expected elution time for tryptophan, small amounts of tryptophan present in hydrolysates are oxidized under normal operating conditions and are not seen.

The gradient conditions were modified so that the hypochlorite oxidizer pump was cut off immediately following the elution of histidine. Oxidizer flow remained off for the rest

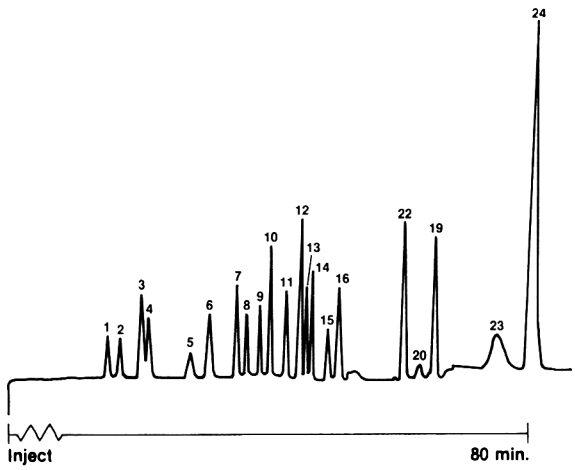

Figure 4. Separation of amino acids by modification of LC method to permit recovery of tryptophan. Tryptophan load 1.25 nmole; all other amino acids 5 nmole each. See Figure 1 for peak identification.

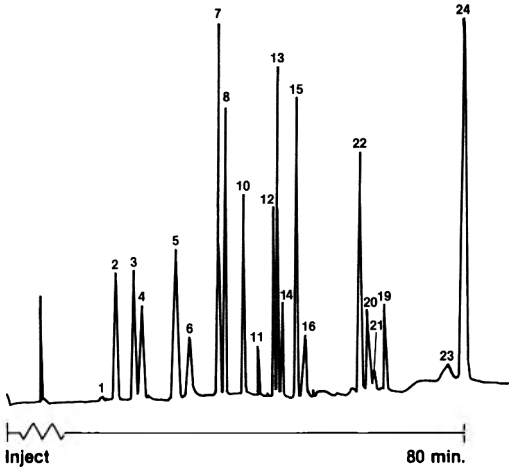

Figure 5. Chromatogram of amino acid analysis of mechanically separated chicken product, showing peak of 3-methyl-histidine. See Figure 1 for peak identification.

of the elution program. This was deemed simpler than trying to turn the oxidizer pump back on following tryptophan elution. We found that oxidizer flow did not stabilize quickly enough to produce smooth elution of arginine. As shown in Figure 4, the arginine response is much larger than that of the other amino acids in the absence of oxidizer flow, but this produced no problems. All amino acids in this case are at 5 nmole except tryptophan, which was set at 1.25 nmole to aid quantitation of small amounts of tryptophan expected in hydrolysates of mechanically separated (species) products. Tryptophan recoveries from the model proteins porcine carbonic anhydrase B (6), pepsin, and egg white lysozyme averaged slightly over 80%, indicating that method performance was satisfactory.

3-Methyl-Histidine

During the past 5 years, much attention has been given to 3-methyl-histidine as a means of estimating the muscle meat content of comminuted meat products. The muscle proteins actin and myosin both contain residues of 3-methyl-histidine. Actin contains 1 residue per molecular weight of 42 000 (7); myosin contains considerably less. Several different methods for estimating 3-methyl-histidine (8–14) have appeared in the literature during the past several years. The most sensitive appears to be an LC technique using a fluorometric detection (14). Once 3-methyl-histidine is determined, muscle meat content of comminuted products may be estimated using a series of computations that involve several conversion factors (15).

Higher muscle meat contents imply increased nutritional value in products. Normal mammalian muscle contains the free amino acid dipeptides carnosine (β-alanyl-histidine) and anserine (β-alanyl-1-methyl-histidine). Another free dipeptide, balenine (β-alanyl-3-methyl-histidine) is found in muscle from whale and pork. It is obvious that methods for estimation of 3-methyl-histidine, especially in pork products, must contain wash procedures that eliminate these peptides. If carnosine is not removed, values for histidine in the essential amino acid analysis will be falsely elevated. Traces of anserine would not interfere in amino acid analysis, but incomplete removal of balenine in pork products could give falsely high values of 3-methyl-histidine, and yield erroneous values for muscle meat content estimations. Each of the 3 mammalian dipeptides anserine, carnosine, and balenine contains a residue of β-alanine, which can serve as a marker

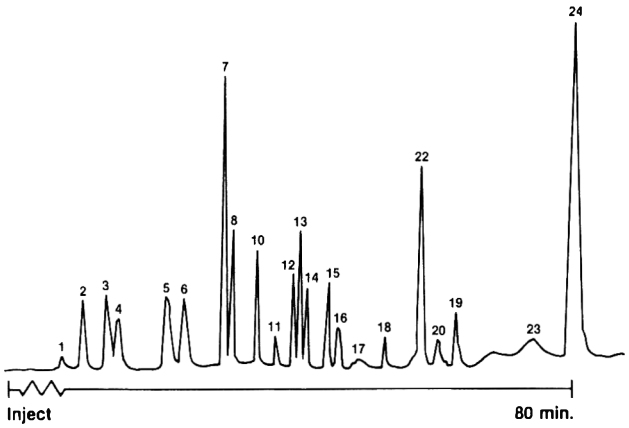

Figure 6. Chromatogram of amino acid analysis of mechanically separated pork product, showing peaks of β-alanine and 1-methyl-histidine. See Figure 1 for peak identification.

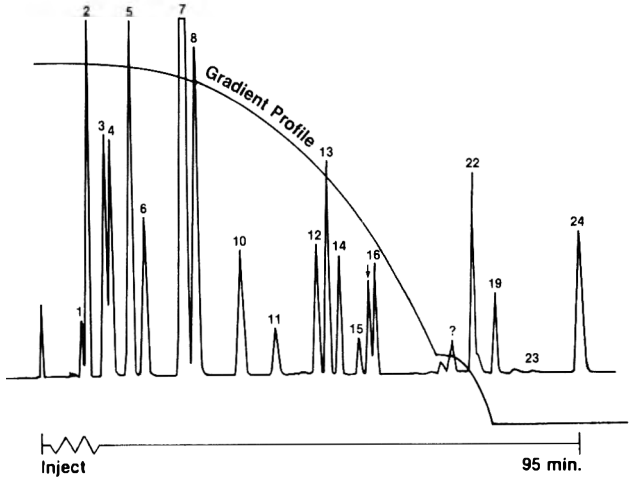

Figure 7. Chromatogram of amino acid analysis of mechanically separated chicken product containing degradative amine contaminant. Arrow indicates contaminant peak in valley between tyrosine and phenylalanine. Separation by gradient extension. See Figure 1 for peak identification.

for incomplete dipeptide removal. Amino acid analysis can also serve as a method for muscle meat content estimation if 3-methyl-histidine is well separated in routine analyses.

We searched chromatograms for indications of β-alanine, 1-methyl-histidine, and 3-methyl-histidine. As shown in Figure 5, a chromatogram of a mechanically separated chicken product, and in Figure 6, a chromatogram of a mechanically separated pork product, β-alanine, 1-methyl-histidine, and 3-methyl-histidine are visible and well separated. However, all of the implications of these analyses are not well understood. The pork samples appeared clear of extraneous balenine, but some residual anserine was present. The analyses of all pork samples indicated essential amino acid contents higher than 33%, yet no 3-methyl-histidine was evident. Conversely, the chicken products all showed peaks of 3-methyl-histidine, no peaks of β-alanine or 1-methyl-histidine, yet none of the essential amino acid contents calculated for the chicken samples was greater than 26%. No peaks of any of these 3 amino acids were observed in chromatograms from veal or beef mechanically separated products. The number of chromatograms was not sufficient to draw any firm conclusions about these observations.

Hydroxyproline

Hydroxyproline, because of its presence in collagen, has long been used as a marker amino acid for this protein. In mechanically separated products from red meat animals, hydroxyproline serves as a ready indicator of collagen derived from bone and connective tissue. In chicken products, the situation is complicated by collagen from skin.

Significant correlations have been shown between PER and the levels of the nonessential amino acids hydroxyproline, proline, and glycine (16). Hydroxyproline has been proposed as an analytical predictor of collagen content, and the Expert Work Group (17) proposed using an upper limit for hydroxyproline content of meat and poultry products as a regulatory tool. Although the Expert Work Group proposed an upper limit of 5% of hydroxyproline nitrogen as a percent of the total protein nitrogen, this proposal has since been modified because calculations showed it was somewhat high.

In the interest of using hydroxyproline as an indicator of protein nutritional quality, a colorimetric assay for hydroxyproline was put in use in FSIS laboratories. The method is a combined modification of the International Standards Organization method (18) and another technique known as the Baltimore Spice method (19).

During 1984, a study was undertaken to assess the protein quality of mechanically separated poultry products in production in the United States. Products were from both raw and cooked poultry, from broilers, spent layer hens, and from turkeys. Samples were obtained from 42 separate plants. Essential amino acids were measured in all samples and hydroxyproline was assayed colorimetrically as well. These data are still being analyzed to correlate hydroxyproline content with essential amino acids and protein quality and to attempt to set a more accurate limit on hydroxyproline content.

Importance of Sample Collection

The specifications for composite sample collection prepared for inspection personnel suddenly became of critical importance during the Mechanically Separated Poultry Products Survey. Directions for sample collection written into the original amino acid analysis procedure specify that the sample must arrive at the laboratory frozen. We had surmised earlier that because of the expected high content of proteolytic enzymes in mechanically separated products, samples would have to be kept near freezing to prevent proteolysis and possible loss of essential amino acids. Sometime after beginning the poultry products survey, the FSIS Midwestern Laboratory noted that many poultry product samples were arriving at the laboratory thawed or even partially decomposed. Such problems had never been encountered with mechanically separated products from red meat animals. Although more rigid directions were immediately written to specify that samples be kept frozen during collection and shipment, and the laboratory immediately began monitoring sample temperatures on arrival at the laboratory, the severity of this problem was not realized until a review of amino acid analyses reports revealed some apparent anomalously high values for several amino acids, namely, aspartic acid, glutamic acid, phenylalanine, and tyrosine. Although it was suspected that these high values might be caused by degradative amines that were coeluting, the problem remained as to how to prove it and to possibly correct the erroneous values.

A search of chromatograms finally located a peak of unknown substance eluting in the phenylalanine-tyrosine region, which appeared to shift slightly from sample to sample, sometimes eluting underneath phenylalanine, sometimes under tyrosine. No definitive proof of any extraneous substance could be found in the aspartic or glutamic acid regions.

Extension of the gradient in the latter portion of the elution program finally moved the contaminant substance to the valley directly between phenylalanine and tyrosine. Figure 7 shows an example of this contaminant separation.

Although it is well known that poultry products carry a much larger bacterial load than do red meat products in general, it was not well appreciated how active these proteolytic enzymes of bacterial origin were until this discovery. Work on improving amino acid quantitation methodology continues in FSIS laboratories.

Acknowledgments

Several chemists contributed to this work over a period of years. The author thanks Randy M. Simpson, FSIS, Eastern Laboratory, Athens, GA; Douglas F. Gillard and Stephanie Brooks, Chemistry Division Laboratory Branch; and Fred Mitchell and Jeff Donahue, FSIS, Midwestern Laboratory, St. Louis, MO, for their technical assistance, suggestions, and efforts.

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Determination of Soy in Meat

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A number of methods may be used for determining soy flour in meat products. Highly purified soy products are more difficult to determine because the nonprotein components used to quantify the flour are reduced. Immunoassays have been used to directly measure protein content of soy products. Immunological methods for determination of soy proteins in meat are complicated by changes in the structure of the soy proteins during processing. These changes alter the available epitopes, changing the immunoreactivity of soy proteins. The epitopes available are dictated by the details of the processing. Other workers circumvented this problem by denaturing the soy protein with urea and mercaptoethanol, and then removing these agents by dialysis; whatever the initial protein conformation, all soy samples came to the same final conformation after the denaturing agents were removed. The assay used antibody made against the "renatured protein." These steps made the assay long and laborious. Attempts to develop a rapid assay were complicated by the same protein denaturation problems. Sodium dodecylsulfate gel electrophoresis coupled with immunoblotting may be the best quantitative approach.

Analytical methods are required to regulate the use of soy proteins in meat products. Developing an assay for this purpose seems like a simple problem. Plant tissues contain a number of substances not found in animal tissues and one need only to determine one of these. Or, because soy and beef are phylogenetically very distant and have little immunological cross-reactivity, the development of an immunoassay should be simple. Although these generalizations on the differences between plant and animal tissues are correct, developing an assay for soy protein in meat has proven very difficult because of the many highly purified soy protein preparations, each with different functional properties, designed for a wide range of products.

This paper reviews the kinds of approaches that have been used to develop a method for determining soy in the presence of meat. Our objective is to discuss and speculate on the sources of the difficulties and suggest some approaches that may prove useful. Method details are discussed only as they relate directly to these objectives. We have not attempted to exhaustively review the literature.

Composition of Soybeans

The composition of soybeans has been reviewed by Smith and Circle (1). The principal constituents of soybeans—protein, carbohdyrate, and fat—account for 97% of the dry weight of the whole bean (2). Soy also contains many minor constituents, including phytic acid and saponins. The use of some of these constituents as analytes has also been reviewed (3). The content of the minor constituents changes with frac-

Accepted March 24, 1986.
Presented at the Symposium on Critical Analysis of Analytical Methods for Meat Foods, 99th AOAC Annual International Meeting, Oct. 27–31, 1985, Washington, DC.
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tionation and processing, so these methods are generally not suitable, especially for the determination of isolated soy proteins.

The seed oil is an example of a constituent that might be used for the analysis of soy in beef were it not for the complications mentioned above. Plants produce long-chain polyunsaturated fatty acids such as linoleic and linolenic acids and are the source of these fatty acids in animal tissues. The fatty acid composition of soy fats and lard is compared in Table 1 (4). The differences are striking, but defatted soy flour contains only about 1% fat and edible ham contains 25% fat, so the fatty acid levels of soy are too low to make a significant difference in the overall composition of soy–meat mixtures containing less than 5% soy. The seed oil is usually removed at an early stage of processing to produce a defatted flour, flakes, or soybean meal. This early removal of the oil complicates the use of plant fatty acids for quantitating soy. Pattern recognition analysis of fatty acids has been used to distinguish 2 species of oxen, *Bos indicus* and *Bos taurus* (5), and may be applicable to the identification of soy in beef, but the small relative contribution of fatty acids from the soy is also likely to be a problem here.

Phytic acid, inositol hexaphosphoric acid, is an important storage form of phosphate in soybeans (6). This is a constituent unique to plants, which could be used to demonstrate the presence of plant material in meat. The concentration of phytic acid in soy preparations depends on the nature and extent of processing. Brooks and Morr (7) reported that in a whole-soy extract, the phytate-to-protein ratio was 0.018; in the 7S protein fraction it was 0.012 and in the 11S fraction 0.001. This kind of change with fractionation reduces the utility of phytate as a quantitative measure of soy in meat. Phytic acid may be an excellent qualitative marker because the presence of any amount would signal the presence of plant material. Raboy et al. (8) found a significant correlation between phytate and protein levels in an analysis of 38 soy lines. This is interesting because soy protein levels vary among plant lines, and phytate could be used to estimate total soy protein in soy flours or in meats containing soy flour.

Phytic acid is chemically important because it has a high charge density. Inorganic phosphate catalyzes lysinoalanine formation (9), and the phytic acid phosphates might also act as catalysts. Phytic acid and phytate–calcium complexes interact with many components in food, and these interactions are likely to be important in soy fractionation schemes (10).

Soy contains some minor proteins that have been considered as analytical markers for soy. Soy lypoxygenase, for example, can be quantitated in native soybeans by measuring the enzymatic activity. But, as the beans age, some of the enzymatic activity is lost due to the instability of 2 of the 3 lypoxygenase isozymes. An immunoassay has been developed that can determine the levels of the lypoxygenase proteins even after the enzymatic activity is lost (11). For processed soy products, the enzymatic activity is deliberately destroyed to prevent the production of undesirable tastes and odors that result from lipid peroxidation. The immunoassay should be able to measure lypoxygenase levels, unless the antigenicity of the protein is destroyed by the treatments used to destroy enzymatic activity, e.g., heat or organic solvent exposure.

The soybean lectin can be rapidly purified by adsorption to an affinity column containing immobilized galactosamine (12) or can be assayed directly by a hemagglutination assay (13). An immunoassay using anti-lectin antibodies has also been developed (Siong Wie, Environmental Diagnostics, personal communication, 1985). The lectin survives hexane extraction, but is heat- and acid-labile (12). As is true of the other minor soy constituents, the amount of carryover of the lectin into the concentrate or isolated soy protein fractions has not been studied.

Table 1. Comparison of fatty acid composition of swine and soy fats[a]

Fatty acid	Lard	Soy fat
Palmitic	32.2	7.0
Stearic	7.8	3.5
Oleic	48.0	17.0
Linoleic	11.0	54.4
Linolenic	0.6	7.1

[a] Percentage of total fatty acids (4).

Soy trypsin inhibitor can be determined by a rapid automated chlorimetric method (14). Unfortunately, the trypsin inhibitor activity is also heat-labile. There is added incentive for analyzing for trypsin inhibitor because this protein has been identified as a specific allergen in a patient who had angioedema and urticaria after eating soy. Moroz and Yang (15) demonstrated a specific IgE antibody response to the soybean trypsin inhibitor. Soybean proteins are commonly encountered food allergens (16, 17). Labeling of soy-containing meat products is an important health consideration.

Soy flour contains 0.6% saponins. The saponins are composed of 5 different aglycones called soyasapogenols A through E, which are similar triterpenoid alcohols synthesized from squalene (18). The sugar moieties contain galactose, glucose, rhamnose, xylose, arabinose, and glucuronic acid with an average of 3 moles of monosaccharide per mole of sapogenin (19). An assay for the determination of the sapogenin has been published (20).

Eldridge and Wolf (21) reported that considerable amounts of the saponins are carried over into the isolated soy protein fraction. The extent to which this is true probably depends on the method of purification, but the sapogenins are interesting soy markers because their structures suggest a variety of approaches that might be used for their analysis. The sapogenins are unique constituents of soy.

Soybeans contain a considerable amount of carbohydrate. Eldridge et al. (22) reported that the average carbohydrate content of defatted soy flours is 27%. Approximately one-fourth of this, or 7.6% by weight, is galactose and 2.4% is arabinose. Thus, galactose and arabinose account for one-third of the soy carbohydrate. Morrissey et al. (23) have taken advantage of this by developing an assay for soy that uses the enzyme galactose dehydrogenase. The enzyme oxidizes galactose with the reduction of NAD to NADH; the reaction is monitored spectrophotometrically by measuring NADH production. The amount of NADH produced is proportional to the amount of galactose and arabinose present after acid hydrolysis of the defatted meat–soy sample. The amounts of galactose and arabinose in beef and in soy flour, concentrate, and isolate, estimated from ref. 23 by multiplying μmole data by 180 and dividing by 1000, are as follows (mg/g): beef 0.11; soy flour 121.0; soy concentrate 109.0; soy isolate 12.2.

Most of the galactose and arabinose probably comes from the arabinans and arabinogalactans present in soy in the cell walls (24). The galactose present in the saponins is negligible, accounting for only 0.07% of the weight of the defatted flour (19). The galactose in the oligosaccharide fraction accounts for about 28 mg galactose/g flour, and the remaining galactose and arabinose are likely to have arisen from the cell wall fraction. The storage proteins, glycinin and β-conglycinin, contain no galactose. This would explain the much lower

galactose concentration in the soy protein isolate fraction. But, even in soy isolates, the galactose plus arabinose concentration is 100 times higher than the concentration in beef. Thus, in beef containing 1% isolated soy protein, the expected galactose plus arabinose concentration would be twice as high as the background. The measurement of galactose and arabinose may be an excellent method for screening for soy products in beef, although other plant materials such as spices also contain high levels of galactose.

Near infrared reflectance analysis (NIRA) has some applicability to the determination of soy in meat. The sample must be very uniform, so sample preparation is a critical step. The NIRA technique is not sensitive and is not reliable for detecting differences in amino acid composition. The method would probably detect OH overtones related to the increased carbohydrate content of soy. If this is true, the method would be similar in capability to the galactose assay (23). The method could be used for flours and concentrates, but not for isolated soy products. Texturates might prove to be especially difficult because, for comparison, all texturates would have to be ground to the same particle size. Particle size is one of the primary factors controlling reflectance in NIRA analysis.

The amino acid differences between soy and beef are not divergent enough to be used for quantitation. Meat does contain some methylated amino acids which can be used to quantitate the beef protein. The amount of soy protein can then be determined as the difference between the amount of beef protein and the total protein. Several methods have been described for quantitating the methylated amino acids (25, 26). This approach for measuring beef is satisfactory, but it is not ideal for the determination of low levels of soy in the beef. Determining the soy by this method requires that a small amount be determined as the difference of 2 large numbers. Other problems arise in samples containing offal or connective tissues, and are discussed by Jones et al. (26). Also, the method provides no positive identification of the soy itself. A direct method is more desirable. An advantage of the use of methylated amino acids is that the method can be used for all meat substitutes, not just soy.

Soy Protein Analysis

The most desirable analyte for the determination of soy in meat is the soy protein itself; this is the critical ingredient representing greater than 90% of the weight of isolates, 70% of the weight of concentrates, and 50% of the weight of soy flours (1). The amount of soy protein present in a soy–beef mixture is difficult to quantitate. Several of the approaches to this problem are discussed below.

Soybeans contain 2 principal storage proteins, glycinin (11S protein) and β-conglycinin (7S protein). Together these proteins account for 70% of the protein in the beans. The remaining proteins are cellular enzymes and structural constituents. The storage proteins are distinguished by their high content of glutamic acid and glutamine and by their relatively low content of sulfur amino acids.

The structure of glycinin, the 11S storage protein, has been reviewed by Nielsen (27). Glycinin has 6 subunits, each containing an acidic and a basic polypeptide chain linked by a disulfide bond. Each subunit is coded by a single messenger RNA, and the acidic and basic polypeptides are formed by post-translational cleavage of the original translation product. The complete amino acid sequences of the acidic and basic polypeptides and the sites of the sulfhydryl cross-links have been determined (27). The molecular weights of the acidic polypeptides are about 37 000, and of the basic polypeptides about 20 000. The molecular weight of the assembled glycinin molecule is approximately 350 000.

β-Conglycinin has a molecular weight of 175 000 and is made up of 3 subunits designated α, α′, and β. The 6 isomers of β-conglycinin differ in subunit composition ($\alpha_2\beta$, $\alpha\beta_2$, etc.). β-Conglycinin contains 5% carbohydrate (28, 29).

Immunoassay of Soy

There is considerable homology among the seed storage proteins of a number of legumes (30, 31), but because of the phylogenic distance from animal proteins, little cross-reactivity between soy and beef is expected. The immunological distinctness of the soy proteins makes the immunoassay approach to soy analysis attractive.

The antigenicity and immunoreactivity of glycinin were studied by Catsimpoolas et al. (32) and Moreira et al. (33). Both groups found that antibody prepared against the native 11S protein did not react with the subunits. Catsimpoolas demonstrated that heating the native 11S protein at 70°C reduced the number of immunoreactive sites. Heating above 80°C resulted in gross conformational changes and the extensive loss of gel precipitation and complement fixation activity. Polyacrylamide gel electrophoresis demonstrated that heating reduced the amount of 11S glycinin and increased the amount of lower molecular weight proteins. Some protein not accounted for by the gel densitometry measurements was assumed to have been higher molecular weight aggregates excluded from the gel.

Moreira et al. (33) studied the relative antigenicity and immunoreactivity of native glycinin and of the purified acidic and basic polypeptides obtained from glycinin. The antibody prepared against native glycinin reacted only weakly with the acidic and basic polypeptides in gel precipitation experiments. Antibodies against the acidic and basic polypeptides did not react with the native glycinin. Antibodies against the individual acidic peptides cross-reacted with other acidic peptides to varying degrees, but did not react with any of the basic peptides. The antibody to acidic peptide A3 was the most general in that it showed the best reactivity with the 4 other acidic peptides.

The immunoreactivity of β-conglycinin is different from glycinin in that determinants are present on each of the subunits as well as on the native 7S protein (29). β-Conglycinin that has been denatured in the presence of 6M urea refolds when the urea is removed and regains its immunoreactivity (28). Therefore, from the immunological point of view, β-conglycinin is more resilient than glycinin. This is mainly because epitopes are present on both the associates and the dissociates. The carbohydrate was eliminated as an important determinant because Thanh and Shibasaki (28) found that glycopeptides derived from β-conglycinin did not interfere with the immunoreactivity of the native protein. Part of the immunological resiliency may reflect the more favorable entropy of renaturation resulting from the lower molecular weight of β-conglycinin.

To develop an enzyme immunoassay for soy, we attempted to prepare a polyclonal antibody that would recognize a broad spectrum of soy determinants. We used a commercial isolated soy protein preparation. To get antibodies to both the native 11S protein and to the subunits, we heated the soy antigen solution to 85°C for 10 min, hoping to create a mixture of the native protein and the subunits. Our objective was to produce antibody to as many epitopes as possible so that changes in the ratio of 7S to 11S or changes in association

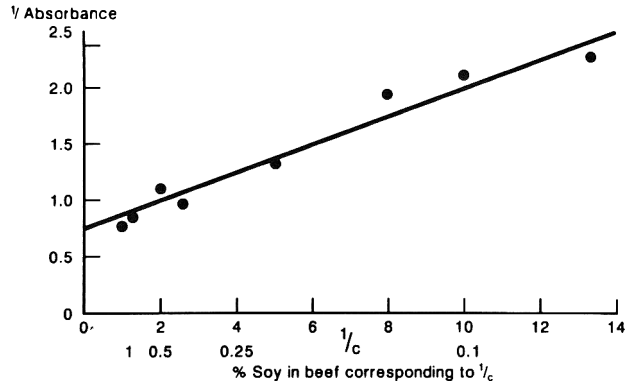

Figure 1. Standard curve: immunoassay for soy in beef. Samples of ground beef and of ground beef mixed with 1% isolated soy protein were each mixed thoroughly in a plastic bag with twice their weights of water. The mixtures were allowed to settle for 5 min at room temperature and the supernatant solutions were decanted and used in the assay. Solutions containing less than 1% soy were prepared by mixing the desired proportions of the control and 1% soy supernates. The beef–soy mixtures were incubated in Gilford cuvet wells, the wells were washed, and antisoy antibody horseradish peroxidase conjugate was added. After 10 min incubation, the wells were washed and the peroxidase substrate, ABTS, was added. Absorbance at 405 nm was measured 7 min later. Absorbance at 405 nm is proportional to the amount of antibody conjugate present which is proportional to the amount of soy protein bound to the wall of the well.

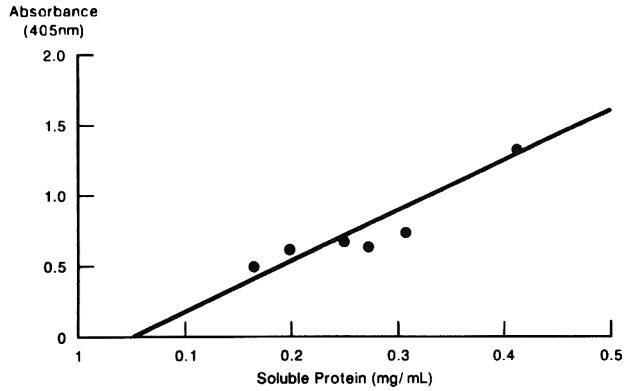

Figure 2. Immunoassay responses of commercial soy preparations. 0.2 g of each of 6 commercially available soy preparations were added to 20 mL 0.1M Tris buffer, pH 8.1. The suspension was mixed, allowed to stand 15 min at room temperature, mixed again, and centrifuged to remove the undissolved material. Gilford wells were coated with each supernatant solution, and the amount of soy protein bound to the plastic was determined with the rabbit antisoy conjugate. Protein concentrations in the supernatant solutions were determined from absorbances at 280 and 260 nm.

and dissociation would not affect our results. The antibodies produced in this way were affinity-purified from rabbit antiserum, conjugated with horseradish peroxidase, and used in the assay.

The enzyme immunoassay was suitable for the quantitation of soy protein which could be extracted into pH 8.1 Tris buffer from raw ground beef. Soy–beef extracts were incubated in polystyrene Gilford® cuvets. The soy protein was bound to the plastic wall in relation to its concentration in the soy–beef mixture. After the cuvet was rinsed, the bound soy protein was determined by measuring the amount of antisoy antibody conjugate that bound to it in a subsequent incubation. After substrate addition, the absorbance increased at 405 nm in proportion to the amount of soy present.

Some data are presented as the linearized reciprocal form in Figure 1. The reciprocal form is based on the Karush equation (34) and can be understood intuitively by considering that, as the amount of soy protein increases, the soy-binding sites on the wall become increasingly saturated. The intercept is the reciprocal of the maximal absorbance, and the slope is the reciprocal of the maximal absorbance times the association constant. The data shown are linear with a correlation coefficient of 0.97. As little as 0.22% soy can be detected in beef when the limit of detection is defined as the amount that differs from the blank by 3 standard deviations (35).

More highly processed products such as texturates formed by hot extrusion are difficult to assay using the immunological approach because of the secondary, tertiary, and quaternary structural changes caused by processing. Higher-order aggregates may also form. These changes cause decreases in the epitopes available on the surfaces of the soy-protein molecule for interaction with the antibodies. As the soy protein is denatured, some epitopes are lost, some are changed, and at the same time the proteins become less soluble. The change in immunoreactivity with processing is caused both by changes in solubility and by decreases in epitope availability. The effect of solubility is shown in Figure 2. The absorbances in the immunoassay of a series of commercial soy preparations are plotted against the concentrations of the soy solutions. Assay absorbance is roughly proportional to the protein concentrations.

Reductions in solubility reduce the immunoassay response. The effect of changing epitopes was shown by preparing a group of solutions from 6 different commercial soys diluted to the same protein concentration, 5 μg/mL. Immunoreactivity, measured as absorbance at 405 nm, was 1.127 for the soluble soy isolate, and for the soy texturates: B, 0.755; E, 0.777; F, 0.743; G, 0.598; K, 0.522. Here, the differences in immunoreactivity reflect differences in the availability of epitopes because all the soys were assayed at the same protein concentration. These values show that the texturates produced about half of the immunoassay absorbance of the soluble "native" protein. Significant differences in reactivities among the texturates themselves can also be seen. Although differences are only about 20–40%, for quantitation purposes they are too large.

This decrease in immunoreactivity *per μg of protein* is caused both by a reduction in the number of epitopes and by changes in the avidity of the antibody for the remaining epitopes. Reciprocal plots of $1/A$ vs $1/C$ for the soluble soy preparation and for the texturate G indicated that the "K" value for the texturate was less than K for the soluble preparation by a factor of 3. K obtained from the slopes of the reciprocal plots is analogous to the affinity constant, but is more complicated because differences in the ability of the 2 soy preparations to bind to plastic are also contained in the constant. K does reflect clear operational differences in the immunoreactivity of the 2 soys.

The differences in immunoreactivities among the soy products, discussed above, can be explained by thinking of soy protein as polymorphic; there are many pockets in the free-energy surface, and the paths between pockets are kinetically determined by the conditions of the soy processing. Each of the many possible configurations of the protein has different sets of epitopes available to the antibody. A monoclonal antibody to soy is unlikely to be successful in an assay because only a small subset of the texturates would have one particular epitope exposed. In most of the texturates the epitope

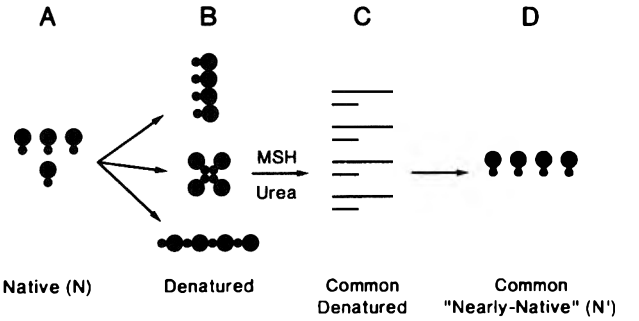

Figure 3. Schematic of texturization-renaturation cycle.
Native soy protein is represented on the left (A). By various processes differing in pH, temperature, etc., the native soy is denatured to form texturates or other less soluble (low NSI) products (B). Differences in higher-order association are depicted. Treatment of the texturates with urea and mercaptoethanol (MSH) separates subunits and opens disulfide bonds, converting them to the denatured open-chain form (C). Removal of the denaturants allows the chains to refold to a common more-nearly-native structure (D).

would be buried. Polyclonal antibodies made in response to a variety of texturized soy proteins are likely to bind to a wider repertoire of soy epitopes. But even if all the epitopes are represented, the decreased solubility of the texturates still presents a problem because only solubilized proteins are able to react with the antibody in most immunoassay formats.

Hitchcock and Griffiths et al. (36, 37) made a very clever attempt to solve the polymorphism and solubility problems. The situation is depicted in Figure 3. Native soy protein is shown on the left of the diagram as the most tightly coiled form of the protein. Native soy can be texturized over a number of paths leading to different conformations and to different degrees and kinds of associations. Each conformation or higher order associate is dictated by the kinetic paths favored by the texturization process. The figure attempts to depict changes in tertiary and high order associations and is entirely speculative. We know that each of the texturates has a different set of epitopes exposed to the antibody and a different solubility. To solve this problem, Hitchcock et al. (36) dissolved all of the soys in a solution of mercaptoethanol and urea. Mercaptoethanol breaks the covalent disulfide bonds between protein chains. Urea, among other things, disrupts internal hydrogen bonds important for maintaining secondary and higher order structures. In the presence of urea and mercaptoethanol, all of the texturates are brought to a common denatured conformation which is stabilized by the presence of the urea. As the urea is removed, all of the once-texturized proteins come to the same nearly native conformation, N′, by traveling over similar kinetic paths. Antibody was made to the renatured protein. Now, if all the soy samples are treated the same way, by dissolving the soy protein in urea–mercaptoethanol solutions, all samples can be brought close to the same renatured configuration, N′. In the N′ form, the antibody could be expected to react with most soys. In fact, most of the renatured soys reacted with the antibody to native proteins, providing evidence that the renatured structure, N′, is immunologically similar to the native structure, N. Unfortunately, the reactivities of different soy products were still too divergent to allow good quantitation.

The different extents of reaction with different soy products might mean that the common denatured state is never really reached. The conditions may not have been suitable for dissipating all of the forces contributing to the stability of some of the texturate conformations. Some of these forces may have been nonsulfhydryl covalent cross-links which form during soy processing. Even though cross-link formation is not extensive, cross-links could act as "grooves in the free-energy surface" which assure that part of the original texturate configuration is retained. Rotmans and Scheven (38) have shown that cross-linked proteins have a higher affinity for the plastic wells and may be selected for in the immunoassay. Lysinoalanine cross-links have been demonstrated in processed soys. These links form in a very alkaline environment, but their formation is also catalyzed by inorganic phosphate at pH levels closer to neutrality (9). Other covalent changes reviewed by Peng et al. (39) should be considered.

Ahern and Klibanov (40) have shown that the deamidation of asparagine residues is an important contributor to the thermoinactivation of egg-white lysozyme at 100°C. At pH 6, the rate of deamidation was sufficient to account for the rate of inactivation. The replacement of neutral asparagines with negatively charged aspartic acid γ-carboxyl groups for the neutral asparagine groups in extruded soy products could also explain why all the texturates do not return to the same state. Differences in the extent of deamidation might also change the path of renaturation. Deamidation is a covalent modification that would not be detected in sodium dodecylsulfate (SDS) gels. Whatever the cause, some differences in the immunoreactivities of texturates persist after denaturation and renaturation.

Table 2. SDS-gel electrophoresis methods for determination of soy in meats

Step	Molander (42)	Lee et al. (41)
Cooking sample	108°C for 1 h 104°C in center	100°C for 1 h <100°C in center
Defatting sample	16 h Soxhlet acetone	homogenize in 10 vols acetone 3 times; dry at 60°C
Extraction of protein	100 vols 3% mercaptoethanol, 3% SDS, 1 h room temp., then 100°C for 30 min	75 vols 1% mercaptoethanol, 3% SDS; boil 15 min
Electrophoresis	slab gel, 12% acrylamide, 0.16% SDS	disk gel, 10% acrylamide, 0.25% SDS
Recovery from cooked product contg approx. 5% soy	3.6% ± 0.6% (sample contained 4.4%)	5.3% ± 1.6% (sample contained 6%)

Gel Electrophoresis Methods for Soy

Some gel electrophoretic methods make use of the properties of denatured proteins without requiring renaturation. SDS gel electrophoresis has been reported by 2 groups to give reasonable recoveries and quantitation of texturates in cooked products. SDS gel electrophoresis is used for determining the molecular weights of proteins. Ideally, SDS binds to proteins in proportion to their size. The charge densities are then the same, and the separation is based only on molecular weight. Some features of the 2 methods which produced good recoveries of soy texturates are given in Table 2. It is difficult to guess which of the details are responsible for the success of the methods, so we have listed some details in the table. In both methods, the defatted samples were extracted into relatively large volumes of *hot* SDS mercaptoethanol. Samples were not cooked at autoclave temperatures, but the conditions were hot enough to account for most food processing conditions. These methods both deserve more attention. In cooked products containing 5% soy texturates, recoveries were within 20% of the known soy content. These recoveries

probably indicate that covalent crosslinking does not involve more than 20% of the protein in cooked products.

A 2-dimensional computer-analyzed high resolution gel electrophoresis system has been described for the characterization of cell lines and for other biological research (43–45). In extracts of cultured cell lines, the methods can detect and identify, by position, 2000 proteins. The system is quantitative and is commercially available as an analytical service. It uses isoelectric focusing in one direction and polyacrylamide gel electrophoresis in the other. The technique may make it possible to identify special meat or protein additive peptides which could be used as sentinels for the identification of the plant or animal-tissue sources of protein additives. Ultimately this may be ideal for a number of regulatory needs. The system deserves careful examination.

Conclusion

We believe that few methods can compete with enzyme immunoassay for qualitative identification of soy in raw meats. This may well be extended to cooked products by the use of urea–mercaptoethanol systems and renaturation as described by Hitchock's group. However, for a general system that does not require special reagents for each analyte and that can be applied to the quantitation of a wide range of protein additives in different product lines, gel electrophoresis may still be the most promising approach.

Acknowledgment

We are grateful to Darlene Worsham for her help in preparing this manuscript.

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Marine Fish as Source of Protein Supplement in Meat

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For the past 2 decades, a great deal of research has been done in fish technology, particularly in the area of mechanically deboned minced fish. Minced fish is the edible muscle flesh of fish that has been mechanically separated from the bones and skin. Ideally, the product is prepared from a high quality fish and resembles hamburger meat. In its final form, minced fish is used either as an ingredient or as an extender in seafood or in food products that require further processing. On the basis of technological advancements, the National Marine Fisheries Service of the U.S. Department of Commerce and the National Fisheries Institute jointly petitioned the U.S. Department of Agriculture in 1980 to add minced fish at a level of 15% in the meat formulation of frankfurters. This paper explores certain aspects of processing, production, acceptance, and hazard assessment of minced fish ingredients as possible protein supplements in meat and poultry food products relative to this request.

The U.S. annual consumption of edible fishery products is about 4–4.4 billion lb with a per capita consumption of 12–14 lb. Total commercial landing of fish in the United States is about 6–6.5 billion lb with an annual value of 2.4 billion dollars. About 3.5 billion lb of these are edible fish and 2.5 billion lb are used industrially (1). Among the species caught, 68% are Alaskan pollock (*Theragra chalcogramma*); the Pacific cod (*Gadus macrocephalus*) and hake species (*Merluccius* spp.) account for about 10%. A major portion of U.S. fishery products is bought by foreign countries. Poland, the Soviet Union, and Japan are the 3 largest buyers of U.S. fishes. About 1000 species of fish are in the oceans; 250 of these species exist in U.S. waters (2).

The Agricultural Marketing Act of 1946 gave the Secretary of Agriculture the responsibility for effective inspection, certification, and identification of class, quality, and conditions of agricultural products. At that time, agricultural products included fish and shellfish as a food commodity. In 1956, the Fish and Wildlife Act transferred all functions of commercial fisheries from the U.S. Department of Agriculture (USDA) to the Bureau of Commercial Fisheries within the U.S. Department of the Interior.

In 1970, these responsibilities were transferred to the National Marine Fisheries Service (NMFS) under the National Oceanic and Atmospheric Administration (NOAA) of the U.S. Department of Commerce. At present, the NMFS inspection is voluntary and is done on a fee-for-service basis.

Minced Fish Technology

The past 2 decades have seen a great deal of progress in fish technology and processing. Perhaps the most important and major change in fish processing is the development of automated mechanical devices that separate fish flesh from bones and skins; the final product is called minced fish (3). Fish from any species can be used.

With an ever increasing demand for fish products, the use of whole fillets, which are traditionally processed into fish blocks, is not economically feasible because of the incurred waste. Minced fish has great potential for being included in the channels of the American food supply (4) because mechanical separation of fish flesh recovers an additional 10–20% usable flesh. Of course, the amount recovered depends on species, size, and other factors. For example, in rockfish, the yield of fillet is only 30%. By the mincing operation, it is possible to increase the yield and recover another 10–12%. In English sole, yield recovery improves as much as 30% (5, 6).

Figure 1 is a flow chart for processing minced fish. A variety of machines is used. The most common are referred to as drum-type machines, and produce a product closely resembling hamburger in texture. First, the fish are deheaded and gutted; then, intact fish are split in half and pressed against a drum pressure plate or high tension belt. The flesh is squeezed through the drum opening into the interior of the drum, while the bones and skins remain on the outer drum surface and are recovered with a blade scraper. The efficiency of separation and the yield can be controlled by adjusting the tension belt or by using different drums having holes of different diameters. Figure 2 shows the basic operation of the drum-type machine.

Minced Fish Quality Factors

Chemical Composition

Although minced fish muscle is an excellent source of protein, its overall acceptance is lower than for products prepared from fillets. During processing, the cells rupture, and enzymes and nucleic acids are exposed to oxidation. This affects the color, taste, and texture of the minced fish, which becomes more prone to oxidative rancidity and thereby to lowered acceptance.

The protein, moisture, and fat content of fish depends on species, size, sex, age, and seasonal variations. The chemical composition of fish also depends on the part considered. For example, in rockfish species, the protein content of deboned fillet is about 18.6% and the fat content is about 1.6%. However, the protein content of deboned whole fish is abut 15.0% and the fat content is as high as 4.1% (7). Fat in fish is made up mainly of highly polyunsaturated fatty acids with carbon chains exceeding 18 carbons, and may vary from 1 to more than 20%.

The pH of fish muscle varies from 6 to 7 and depends on the species and age of the product (8).

Protein and Nutritional Values

Approximate levels of protein in fish are 22.0% sarcoplasmic protein, 60% myofibrillar protein, and 7% connective tissue stroma. The nonprotein level is about 11–12%. Fish protein has an excellent balance of amino acids and is high in essential amino acids. Its lysine content is higher than that of land animals such as beef, pork, lamb, and poultry. The protein efficiency ratio (PER) of fish is about 3.2 compared with PER values of egg protein. Polyunsaturated fatty acids in fish are high. Sodium content of fish is very low, about 70 mg/100 g (8–10).

Storage Stability

The storage life of frozen minced fish is between 6 and 12 months, depending on the species and fat content of the product (5, 11). Preservation and storage of minced fish is a

Accepted March 24, 1986.
Presented at the Symposium on Critical Analysis of Analytical Methods for Meat Foods, 99th AOAC Annual International Meeting, Oct. 27–31, 1985, Washington, DC.

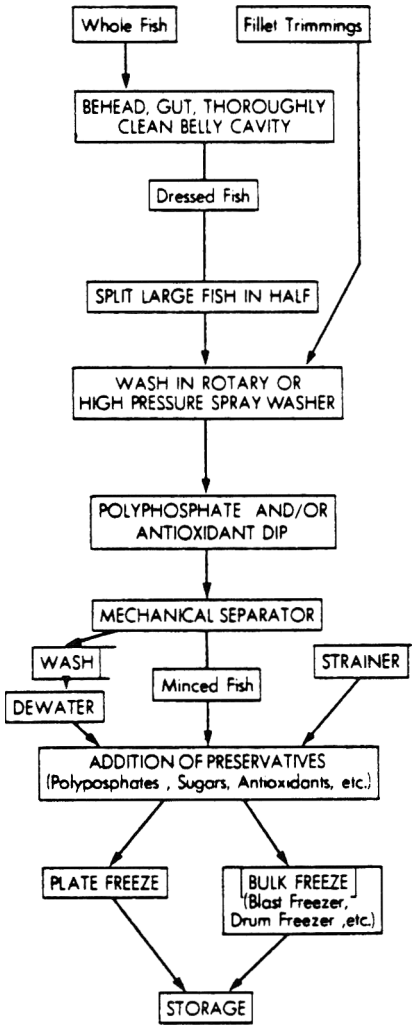

Figure 1. Flow chart for production of minced fish. Reprinted from ref. 3 with permission of UNC Sea Grant Program.

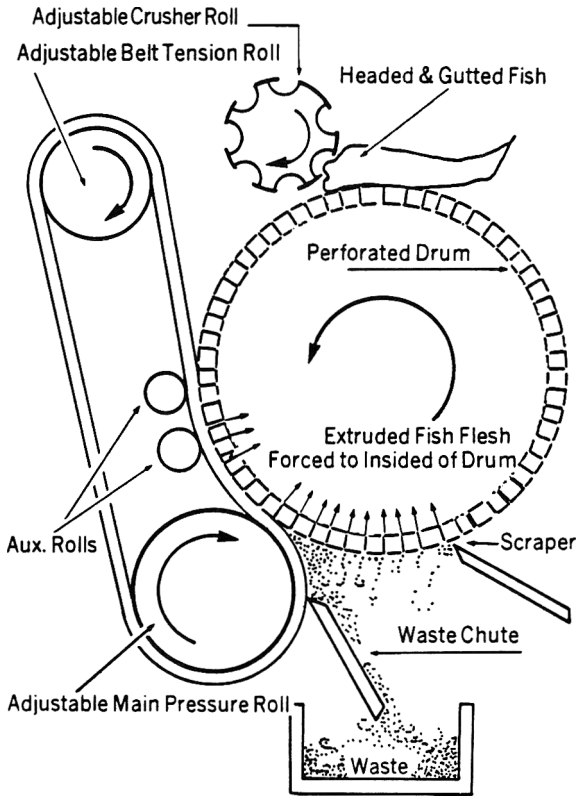

Figure 2. Operation of drum-type mechanical separator. Reprinted from ref. 3 with permission of UNC Sea Grant Program.

problem, because of susceptibility to bacterial spoilage and rapid texture deterioration. Tests show that minced fish in frozen storage generally deteriorates at about twice the rate of frozen fillets. In the cod family, the rapid deterioration of texture in frozen storage is due to the presence of the enzyme system that converts trimethylamine oxide to dimethylamine and formaldehyde. This enzyme is located in the dark flesh and kidney tissue. It is important, in most species, to remove the backbone in order to remove the kidney and thus control rapid texture deterioration. Rancidity is also a problem in frozen minced fish, and tests have indicated that addition of antioxidants such as BHT (butylated hydroxytoluene) and BHA (butylated hydroxyanisole) is very useful in its prevention.

Product Development and Acceptability

The use of mechanically separated fish flesh has stimulated new product development ideas. For example, through research and marketing over the past 10–20 years, products such as fish sausage and fish patties have been developed and marketed in Japan, Canada, and many European countries (12–17).

When the flesh of fish and terrestrial animals is examined, the difference in appearance and texture suggests great differences in the composition of the muscle of the 2 groups of animals. For example, fish muscle is considerably more tender than that of beef or poultry; it has an obviously different flavor; and it responds differently to different kinds of treatment. Fish has about the same protein content as beef but protein and fat content varies more among various species. Fish has much less connective tissue compared with beef, and therefore requires no aging and only a minimum cooking time to produce a tender, succulent product. Although the basic muscle structure of all animals, including fish, is alike, there are great differences in functional properties of muscle among species of fish. For example, pelagic fish are generally soft, and demersal species are firm in texture. By mixing flesh of different species of fish, a minced fish product with desirable functional properties can be produced (18).

Since 1968, new research has been undertaken at different NMFS Utilization Research Centers. The research has included a study of the characteristics of minced fish prepared from various species, and the commercial potential of minced fish as a protein ingredient in processed foods. For example, numerous researchers have used fish as a partial replacement for beef muscle in sausage products (12, 15, 17, 19, 20). In producing new food products, the meat industry should have assurance that minced fish ingredient is available on a continuing basis, and that it results in a food product of universal quality.

In 1976, NMFS prepared a number of samples using minced fish, soy, and poultry combinations, and the products were evaluated by the USDA Food and Nutrition Service (14, 15). In more recent research efforts (19), NMFS has sponsored independent product development at American Bacteriological and Chemical Research Corp. (ABC), Gainesville, FL. Frozen blocks of Alaskan pollock, an underutilized species that contains a high percentage of protein and low fat, was used for the product development. Pollock fillets at levels of 20, 30, and 40% were used in fish/meat sausage link for-

mulations. The results have shown that the links containing fish had lower fat content when the percentage of fish was increased. Thiobarbituric acid (TBA) values (a measure of oxidative rancidity) were in an acceptable range and were comparable with those for the control test products.

When the franks containing minced fish were compared directly with the corresponding all-meat products, panelists noted a slightly "different" but acceptable taste. The apparent flavor difference was not noted as a fishy flavor. A somewhat softer texture was also initially observed with the franks containing higher levels of fish. When franks containing fish were compared for texture with some commercial brands, the test franks were rated the same or slightly superior in some cases. No major differences were noted in the shelf-life of either vacuum-packed product. The study showed that substitution of fish for beef provides the most economical use of fish protein, resulting in a lower ingredient cost and a product with lower fat content.

In another study, NMFS completed a series of acceptability tests using 10% Alaskan pollock to extend frankfurters and meat patties for the USDA school lunch program (15, 21). The all-meat patties and franks and the experimental products were served as hamburger or as the main dish in the school lunch, which included salad, milk, and dessert. Four tests were scheduled so that the regular all-meat franks or patties were used on different weeks and the experimental products with fish were used on succeeding weeks. The level of acceptability or consumption of experimental meat patties and franks was determined by recovering the uneaten portions from the lunch trays. From 492 all-meat franks served, only 2.8% was recovered; from 491 franks containing fish served, 3.3% was recovered, indicating no significant difference in acceptability of the 2 products. In other studies, similar results were obtained for franks and patties containing fish (5).

Hazard Assessment

Processing

The processing of minced fish allows the commingling of whatever happens to be on or inside the fish. These may be microorganisms, blood, viscera, or extraneous materials, which may eventually become part of the finished product and never be removed. The fish is washed immediately before processing, sometimes with chlorine water. To improve the quality of minced fish, it may also be washed with water. This removes color pigment, soluble protein (up to 25%), and some fat and flavoring materials. In dark muscle fish, the minced fish is washed to remove blood and fat. Sometimes washing lowers the microbial count; it most certainly affects the texture, flavor, and overall acceptability and stability of the product (22–24).

Microbial Contamination

Either prior to or during processing, minced fish may be contaminated by the natural occurrence of microbes in the raw material or during handling of the product. The main concerns are the usual microorganisms of public health significance, such as *Escherichia coli, Staphylococcus, Salmonella,* and *Shigella* (25). Marine pathogens such as *Vibrio parahaemolyticus* sometimes become a problem. During deboning, the cellular content of fish, which is mainly made up of amino acids, is released, and at a suitable temperature this provides a good medium for microbial growth. To alleviate this problem, an extra clean environment and equipment are needed for processing (23, 26–28).

Parasites

It is generally known that from time to time all fish species may have parasites. Although parasites generally are not a significant health problem, from an aesthetic standpoint, it is desirable to have no parasites in the finished products. So far, methods for the objective determination of the number of parasites in minced fish have not been developed (29).

Stability

The most obvious factor in considering stability is rancidity. The process of mincing mixes fatty materials into the flesh and also results in exposure of the particles to air, increasing the rate of oxidation. In species with darker meat, hemoglobin and myoglobin pigments act as catalysts for oxidative reactions.

The large number of *Gadoid* or cod family species have a different stability problem. In these species, trimethylamine (TMA) oxide is at a high level, and probably is enzymatically converted to dimethylamine (DMA) and formaldehyde. This results in a low water-holding capacity and poor texture.

To increase stability, minced fish is washed to remove color pigments, enzymes, TMA, and fat, and then BHT or BHA is added to prevent rancidity (24, 30, 31).

Toxic Substances

Another aspect of stability is histamine formation. During the spoiling process of fish flesh, some microorganisms such as *Proteus morgani* are capable of breaking down the amino acid histidine and, through a decarboxylation process, this amino acid is converted to histamine. Histamine is toxic to some individuals. This is a more severe problem in scromboid species such as those of the tuna, sardine, and mackerel families. In quality control for processing of minced fish, baseline data should be established for each species to test for histamine. Particularly whenever fish is suspected of having been subjected to elevated temperatures for any length of time, it is important to check for histamine content. Saurine is another toxic substance that enhances the physiological effect of histamine. The name comes from an English word for "saury," which in dried form can cause toxicity (29, 32).

Another toxic organism, *Ciguatera,* is found during occurrences of red tide in shellfish beds, caused by a marine organism called *Gymnhodium breve* (a dinoflagellate). Other toxic materials are mycotoxins, such as aflatoxins B_1, B_2, G_1, and G_2 (29), produced by molds.

Pesticides, Microconstituents, and Heavy Metals

Hazard assessment of minced fish should include analyses for pesticides which are covered under food additive laws of the U.S. Food and Drug Administration (FDA) and the U.S. Environmental Protection Agency. FDA sets guideline tolerance levels for chemicals such as aldrin, DDT, the polychlorinated biphenyls, and mercury.

NMFS has carried out extensive research on 15 microconstituents in 204 species of seafood. The data show levels of 300 ppm calcium, less than 0.1 ppm cadmium, 100 ppm fluoride, and less than 0.1 ppm lead (29). So far there is no guideline for heavy metals except mercury, which is set at 0.5 ppm by FDA.

Bone Particles

The typical bone content of minced fish is in the form of bone fragments. Bone fragments may be soft and pliable and

not easily detected when the cooked product is eaten, but some bone in minced fish may also be sharp, pointed, rigid, and potentially harmful. Bones that feel rigid when pressed axially between the fingers are harmful and should be eliminated from the product (33).

The allowance for bone material in the U.S. Department of Commerce grade standards for minced fish is based on a given number of bone fragments per 5 lb product. Bone is defined as any objectionable bone, or piece of bone, that is ¼ in. in length or longer and is sharp and rigid. Perceptible bones shall also be checked by their grittiness during the normal evaluation of the texture of the cooked product. Bones, as defects, are prorated on a 5 lb sample basis; (*1*) slight, 1–2 bones per 5 lb sample; (*2*) moderate, 3–4 bones per 5 lb sample; (*3*) excessive, over 4 bones, but not to exceed 10 bones per 5 lb sample (34).

The frequency of occurrence of bone fragments in minced fish depends largely on the size of the final extrusion openings. Short bone fragments appear in the minced fish when the bones are aligned transversely across the extrusion opening and are sheared off and pressed through during operation. Longer fragments appear when, by chance, bones are aligned axially with the extrusion openings and are pressed through before being sheared off.

The presence of bone in any processed food containing minced fish could have a significant effect on consumer acceptance of the product. An extensive study was performed on the average number of bones for 4 bone size categories after deheaded and gutted fish were passed through a Bibun flesh separator (7 mm perforation) under minimum pressure and again under the same pressure. The samples were tested by an experienced sensory panel, and panel sensitivity was compared with the number of bone particles detected objectively. Using the "flotation method" to detect bone fragments on the first run of deheaded and gutted rockfish, 24.8 bone particles per lb were detected. However, the sensory panel detected only 1.7 bone particles per lb or about 7% of those present. These results show that small diameter bones, even when contacted during chewing, are difficult to sense by tacile means. It should be pointed out that in minced fish, bone particle content appears to be a function of processing methods and machinery used, not of species (7, 19).

Summary

Mechanically deboned minced fish is the edible flesh of fish, which varies in color, texture, taste, and storage stability. Mechanical separation of flesh in fish is an economical and efficient process which results in a higher yield than the filleting process. The process permits mincing different species with different functional properties to produce a uniform product with a desirable ingredient profile. In its final form, minced fish could be used as an ingredient or as an extender in prepared foods requiring further processing. Minced fish as a food ingredient has many different possibilities and could be used as a source of protein food supplement in the U.S. food channels.

With regard to the potential for its use in meat and poultry food products, USDA has statutory authority and responsibility for assuring the public that meat and poultry products are wholesome, not adulterated, and properly labeled, packaged, and marked. Therefore, for USDA to consider the use of minced fish in meat and poultry food products, attention must be focused mainly on areas such as species, residues, contaminants, sanitation, and bone fragments (number and particle size), in addition to the name and labeling of the products.

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Rapid Methods for Determination of Meat Composition

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Rapid analytical procedures are needed to determine the total protein, moisture, and fat content of meat and poultry products. During the past 5 years, the U.S. Department of Agriculture (USDA) has been studying various methods involving instrumentation that test for these constituents either in combination or separately. The studies are initiated on request of the Department or the instrumentation manufacturer and are conducted at the manufacturers' facilities using 2 sets of samples preanalyzed by conventional means. One set, with values, is used for calibration purposes, the other set is tested as unknown samples. The resultant data are evaluated statistically vs the conventional test results. Most studies show the usefulness of these rapid tests for product quality control, but some fall short of regulatory requirements because of unacceptable bias or variability. Typical within-product standard deviations obtained in rapid methods instrumentation tests have ranged from 0.47 to 0.67 for percent total protein, 0.73 to 1.71 for moisture, and 0.41 to 1.14 for fat. For conventional methods, the acceptable USDA performance criteria for repeatability are standard deviations of less than 0.24 for protein, 0.46 for moisture, and 0.63 for fat. Improvements in instrumentation are being made and studies continue.

The Chemistry Division of the U.S. Department of Agriculture, Food Safety and Inspection Service (USDA-FSIS), Science, evaluates instruments and rapid techniques for the determination of moisture, protein, fat, and salt in meat, poultry, and meat and poultry products. In this paper, the reviews and studies are considered as 2 groups, those that were performed prior to 1980, and those performed from 1980 to 1985. Tables 1 and 2 summarize investigations during those 2 periods. The instruments and techniques listed in the 2 tables are not exhaustive; there may be others that were not evaluated because of constraints on Chemistry Division resources or because we were not aware of them.

In the tables, the term "rejected" in the column headed "Recommendations" needs explanation. Each instrument/technique can be viewed from 2 different perspectives: (*1*) Can or should it be used for quality control purposes within a meat processing plant, and (*2*) is it appropriate for regulatory purposes? The term rejected refers only to the latter. It is useful to remember that technical improvements are continuously being made and that instruments studied several years ago may deserve reevaluation. Performance criteria should be applied in determining acceptability for quality control purposes or for regulatory work.

In-plant quality control tests are often designed to give the user a very rapid technique for evaluating an intermediate step in the process or the finished product. Usually, very few tests per day are performed. Accuracy requirements can range from a yes/no answer at a specific analyte level, to values within a range of levels, to values that must meet specific standards for repeatability and bias. Plant management decides on acceptability and performance criteria for its own quality control tests.

Regulatory requirements are much more stringent. Many of these regulatory performance criteria can also apply to in-plant quality control. Regulatory laboratories perform analyses on large numbers of samples every day. Therefore, an instrument/technique that may require only a couple of minutes for each specific test can become so work-intensive if the instrument requires constant analyst attention, that the regulatory analyst cannot perform other mandatory tests. Instruments that require calibrations for each kind of product or each formulation are not practical. Ability to simultaneously determine more than one analyte compensates for labor-intensive techniques.

Sampling is a particularly difficult problem. Some meat samples are difficult to prepare homogeneously. This intro-

Accepted March 24, 1986.
Presented at the Symposium on Critical Analysis of Analytical Methods for Meat Foods, 99th AOAC Annual International Meeting, Oct. 27–31, 1985, Washington, DC.
Mention of a trademark, proprietary product, or vendor does not constitute a guarantee, warranty, or endorsement of the product by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products or vendors that also may be suitable.

Table 1. FSIS rapid methods investigations before 1980

Analysis	Instrument/technique	Manufacturer	Recommendation
Protein	AutoAnalyzer/1, automated colorimetric with helix digestion; 2, with block digestion	Technicon	1. official AOAC—(helix) 2. block digestion—collaborative study, results not reported
	Kjel-Foss/automated Kjeldahl	Foss-America (Dickey-john)	official AOAC
	Coleman Model 29A/volume of released nitrogen	Coleman, and Perkin-Elmer	rejected—samples too small
	/conductivity of released ammonia	Leco Corp.	rejected—samples too small
	protein analyzer/dye binding	Baltimore Spice Co.	satisfactory for plant QC, rejected for regulatory use—too variable
Moisture	/Karl Fischer manual and automatic titrations	several	rejected—low bias
	/microwave oven drying	Photovolt Corp., Apollo, Risman, Steele, Lee & Latham	originally rejected, may be covered under official AOAC method **24.A01–24.A03** (1985); satisfactory for plant QC (*proper calibration imperative*)
	Moisturefuge/spin dry toluene extract	Anderson Labs	satisfactory for plant QC
	moisture balance/IR lamp after toluene distillation	Ohous, Cenco	satisfactory for plant QC
	moisture tester/timed rate of evaporation	Brabender	satisfactory for plant QC
	/azeotropic distillation	ARS research paper	accurate, but solvent storage and disposal problems—rejected
	/GC of anhydrous isopropanol extract	published—Canadian Institute of Food Technology	rejected, but might be worth second look
	/capacitance of dioxane extract	—	rejected—not accurate
Fat	/modified Babcock and Paley bottle methods	—	rejected—not accurate above 27%, satisfactory for plant QC up to 27% (may have high bias)
	Banco/rendering	Banco, Anderson Labs	rejected—standard deviation 0.9%
	Univex Fat Analyzer/rendering	Univex	rejected—ground beef only, std dev. 1.6%
	/electrical inductance	EMME (Dickey-john)	whole carcass size, but laboratory size units now available, might be worth second look
	/NMR	—	rejected—too expensive, fast measurement but too long sample preparation time
	Steinlite/electrical capacitance after dichlorobenzene extract	Steinlite	satisfactory for plant QC, std dev. 0.5%
	GMA/IR reflectance	Neotec	rejected—ground, fresh meat only; not accurate above about 26%
	digital fat controller/	Honeywell	no longer manufactured
	/gallium arsenide IR emission	Massie	rejected, std dev. 2%
	/liquid CO_2 extraction	—	rejected—safety problems
	/column elution (9 + 1, dichloromethane–methanol)	—	satisfactory for plant QC
Salt	/specific ion electrodes	several	rejected—matrix interferences (still under study)
	Quantab/paper chromatography	Ames, Miles Laboratories	official AOAC—but not recommended—too variable
Moisture, protein, fat, and ash	Ultra X/IR lamp	Koch	rejected for protein (by difference—not accurate); satisfactory for plant QC for moisture, fat and ash

duces the necessity for analytical samples of sufficient size to ensure representativeness. Regulatory samples are very diversified, and may be anything from a cut of meat to highly emulsified cooked sausages. Acceptability of analytical procedures depends on their ability to meet established performance standards for repeatability and reproducibility regardless of sample type.

In addition, the test must not be biased compared with methods used to develop the regulations. Established standards are those obtained using procedures deemed to be "official." Currently, for meat regulation, these methods are from Chapter 24 of the AOAC *Official Methods of Analysis*, 14th Edition (1984) (AOAC, Arlington, VA). Table 3 shows acceptable performance criteria published by FSIS. Performance standards for these and other tests can be found in the USDA-FSIS, Science, "Chemistry Quality Assurance Handbook." Copies may be obtained on written request to USDA-FSIS-MPITS, Program Training Division, PO Box 1608, Denton, TX 76201. Attn: Distribution Record System.

Some analytical techniques, regardless of the instrumentation, do not appear to be applicable to meat analysis. Karl Fischer titrations have an unacceptable low bias. Nuclear magnetic resonance techniques are not rapid because of the long sample preparation time required, and the instrumentation is expensive. Use of infrared reflectance devices requires special care. These instruments have the advantages of speed, specificity, and simplicity, but if the same analytical sample is remixed slightly before a second reading is taken, that reading often varies too greatly from the original. Techniques using dangerous solvents or those that have waste disposal problems should be avoided, as should instruments designed only for the analysis of powders, grains, liquids, etc., which are not appropriate for meat samples.

Since 1980, 6 extensive studies have been conducted on

Table 2. FSIS rapid methods investigations, 1980–1985

Analysis	Instrument/technique	Company	Year	Recommendation
Protein	P-100 Protein Analyzer/low resolution pulsed NMR	Oxford, New Port	1980	rejected—too expensive; fast readout, but long sample preparation
	/block digestion automated distillation and titration	Tecator, Buchi-Brinkmann, Labconco	1980	official AOAC
	706 Nitrogen-Protein Analyzer/chemiluminescence	Antek, Unimat	1981	rejected—sample too small
	UDY Protein Analyzer/dye binding	U.D. Corp.	1981	may be satisfactory for plant QC; too variable for regulatory analysis
	Carlovera Nitrogen Analyzer	Carlo Erba	1983	rejected—sample too small unless freeze-dried first
	Protein Assay Kit/dye binding	Bio-Rad	1984	rejected—std dev. 3%
	Digesdahl/Nesslerization	Hach	1984	(see text) shows promise, still under study
	Protimeter/IR reflectance	Protimeter Ltd	1984	rejected—for grains only
Moisture	KF-4B Aquameter/Karl Fischer	Beckman	1980	rejected—low bias
	PR-103/pulsed NMR	Praxis Corp.	1981	rejected—too long sample preparation, very expensive
	Compu-Trac/IR moisture oven	Compu-Trac	1982	rejected—too variable
	Moisture Meter Model 919A/IR reflectance	Motomco, Inc.	1982	rejected—for powders and grains only
	Metrohm Model 633/Karl Fischer	Sybron-Brinkmann	1982	rejected—low bias
	LP-15 coupled to PC series balances and GA-40 printers/evaporation	Mettler	1982	rejected—low bias
	Accu-Dry/IR oven drying	Artek Systems Corp.	1982	satisfactory for plant QC
	Moistu-Trac/PM-80/IR reflectance	Berwind	1982	satisfactory for plant QC
	Models CA-50, VA-05 Moisture Measurement System/Karl Fischer	Mitsubishi	1983	rejected—low bias
	Automatic 4586 Moisture Tester/IR reflectance	Forte Technologies	1983	rejected—range 0–40% moisture
	Infra Dry/IR oven	New Brunswick	1984	satisfactory for plant QC
	Model MC-Moisture Computer/capacitance—dielectric constant	Aqua Measure Instrument Co.	1985	follow-up in progress
	Model G8R and G9/radio frequency power loss	Data Tech	1985	for powders and granular samples only, might be useful for future technology
Fat	Rapid dry column/dichloromethane–methanol extract of Celite—sample column	research paper–Maxwell et al., JAOAC, 63, 600	1980	rejected—0.6% high bias; measures total lipids not crude fat
	SFC-900/solid fat analysis	Praxis Corp.	1981	rejected—probably satisfactory for lard, butter, etc.
	Anyl-Ray Fat Analyzer (production line and lab. table top models/x-ray absorption	Anyl-Ray Corp., The Kartridge Pak Co.	1981	satisfactory for plant QC
	DjME-100 Ground Meat Analyzer/IR reflectance	Dickey-john (EMME)	1981	satisfactory for plant QC, ground beef only
	MIRAN-80/IR reflectance	Foxboro	1982	rejected—for dairy products and snack foods only
	Rafatec Extractor, Soxtec HT/ether extraction (Randall)	Tecator	1983	very promising, should be collaboratively studied
	Foss-Let/specific gravity	Dickey-john (Foss America)	1983	official AOAC
	Optec/IR reflectance	Optec	1985	Investigation in progress
Salt	Chlor-o-Stat titration/coulometric titration	Fisk Associates	1982	satisfactory for plant QC
	Dicromat/conductivity	Diamond Crystal Salt Co.	1983	satisfactory for plant QC, requires calibration for each type of product
	NOVA/specific ion electrode	NOVA Biomedical		too variable unless very carefully controlled
	/ion specific electrodes	Several		studies in progress
Fat and moisture	CEM/microwave oven	CEM	1983–1984	official AOAC (generic microwave oven)
Protein, moisture, and fat	Noetec Model 102/NIR reflectance	Pacific Scientific	1983	rejected—too variable, may be satisfactory for plant QC if properly calibrated
	Automatic Meat Analyzer Model FMP-1/microwave oven	Hobart	10/81 1/83	(see text) satisfactory for plant QC if properly calibrated for each specific formulation; rejected for regulatory analysis
	InfraAlyzer 400/IR reflectance	Technicon	5/82	(see text) not acceptable for regulatory analysis
	Super Scan/IR transmittance	Dickey-john	9/81 10/84	(see text) third study in progress—shows promise

Table 3. FSIS criteria for acceptable performance of methods for meat analysis

Component	Standard deviation: Repeatability	Standard deviation: Reproducibility	Acceptable bias
Moisture	<0.46	<0.65	±0.125
Protein	<0.24	<0.32	±0.125
Fat	<0.63	<0.66	±0.125
Salt	<0.16	<0.16	—

Table 4. Mean differences and standard deviations of differences between results obtained using InfraAlyzer and official AOAC methods—1982 study

Product	Sample size	% Protein: Mean diff.	% Protein: Std dev. of diff.	% Moisture: Mean diff.	% Moisture: Std dev. of diff.	% Fat: Mean diff.	% Fat: Std dev. of diff.
Cooked sausage	7	0.32	0.63	1.64	2.26	−0.36	1.22
Ham	24	−1.10	0.75	2.10	2.24	2.05	1.46
Pork sausage	7	−0.18	0.58	−0.14	2.42	−0.18	1.76
Ground beef	7	−0.49	0.51	1.66	0.36	−0.79	1.54

specific rapid test equipment using samples supplied by USDA. These tests were initiated at the request of either the USDA-FSIS or the instrument manufacturer. They were not intended as collaborative studies but only to determine performance characteristics to see if standards could be met and whether further studies might be appropriate. Future requests for such testing will continue to be honored as resources are available, such as the large number of samples required. The findings from the 6 studies follow.

1. Technicon InfraAlyzer—May 1982

Forty-five samples of 4 product types (cooked sausage, ham, pork sausage, and ground beef) were analyzed in duplicate by InfraAlyzer instrumental and official AOAC methods. The instrument was rejected for regulatory analyses because of the large bias compared with official AOAC methods—Kjeldahl (protein), oven drying (moisture), and Soxhlet ether extraction (fat). Also, absolute differences between instrumental and official AOAC method results were as high as 1.4% for moisture and fat (Table 4).

2. Foss America Super Scan—September 1981

Forty samples of 15 product types (ham, pork sausage, pepperoni, franks, bacon, canned corned beef, mortadella, luncheon meat, salami, ground beef, genoa salami, chorizo, bologna, canned picnic, and soppresate) were analyzed in duplicate by Super Scan instrumental and official AOAC methods. The instrumental analyses were considered unacceptable because the overall bias was too large for moisture, protein, and fat, and the standard deviation was too large for protein and moisture analyses (Table 5).

Table 6. Mean differences and standard deviations of differences between results obtained using Dickey-john Super Scan and official AOAC methods—1984 study[a]

Product	% Protein: Mean diff.	% Protein: Std dev. of diff.	% Moisture: Mean diff.	% Moisture: Std dev. of diff.	% Fat: Mean diff.	% Fat: Std dev. of diff.
Canned ham	−0.24	0.43	−0.05	0.68	0.43	0.28
Cooked sausage	0.47	0.29	−1.80	1.16	1.12	0.49
Hamburger	0.63	0.31	−0.36	0.88	−0.45	0.80
Pork sausage	0.35	0.43	0.13	1.45	−0.22	0.96
Smoked ham	0.45	0.51	−1.54	1.14	0.22	0.72
Water-added ham	0.59	0.58	−1.50	0.98	0.24	0.90

[a] Sample size = 16.

3. Dickey-john Super Scan—October 1984

Ninety-six samples of 6 product types (canned ham, cooked sausage, hamburger, pork sausage, smoked ham, and water-added ham) were tested in duplicate by Super Scan instrumental and official AOAC methods. Mean differences and standard deviations of the differences between results for the instrumental and official AOAC methods indicate that the instrument may be acceptable for regulatory purposes with slight modifications and improvements in moisture and fat analyses (Table 6).

Another test of the Super Scan instrument was conducted in September 1985, at Webb Foodlab, Raleigh, NC. Results from that study are still being evaluated.

4. Hobart Automatic Meat Analyzer Model FMP-1—October 1981

Forty samples of 13 product types (ham, bacon, pork sausage, franks, semidry sausage, canned corned beef, luncheon meat, pepperoni, mortadella, prosciuttini, ground beef, bologna, and canned ham) were analyzed in duplicate by the Hobart instrumental and official AOAC methods. The instrument was considered unacceptable because of the large overall bias (Table 7).

5. Hobart Automatic Meat Analyzer Model FMP-1—January 1983

Ninety-six samples of 6 product types (canned ham, cooked sausage, ground beef, pork sausage, smoked ham, and water-added ham) were analyzed in duplicate by the Hobart instrumental and the official AOAC methods. Again, the instrument was considered unacceptable because of the large overall bias compared with AOAC results (Table 8).

6. Hach Company Digesdahl—February 1985

Ninety-six samples of 6 product types (hamburger, pork sausages, water-added ham, canned ham, cooked sausage, and ground beef) were analyzed in duplicate for percent protein by the Digesdahl instrumental and official AOAC (Kjel-

Table 5. Statistical evaluation of 1981 study of Foss America Super Scan instrumental vs official AOAC methods

Statistic	% Protein	% Moisture	% Fat
Instrumental within-product std dev.	0.47	1.30	0.41
Av. diff. between instrumental and AOAC results	0.62	9.01	0.79

Table 7. Statistical evaluation of 1981 study—Hobart analyzer vs official AOAC methods

Statistic	% Protein	% Moisture	% Fat
Instrumental within-product std dev.	0.05	0.73	0.65
Av. diff. between instrumental and AOAC results	1.36	0.60	1.46

Table 8. Mean differences and standard deviations of differences between results obtained using Hobart analyzer and official AOAC methods—1983 study[a]

Product	% Protein		% Moisture		% Fat	
	Mean diff.	Std dev. of diff.	Mean diff.	Std dev. of diff.	Mean diff.	Std dev. of diff.
Canned ham	0.63	0.69	−0.28	0.64	0.49	0.60
Cooked sausage	−1.00	1.4	−1.36	0.84	1.35	1.35
Ground beef	0.32	0.63	−0.47	0.78	0.57	1.58
Pork sausage	−1.11	1.82	0.22	0.70	1.66	2.33
Smoked ham	−2.46	2.00	−2.31	2.2	4.58	1.90
Water-added ham	−1.23	1.48	−1.90	1.05	2.78	1.90
Average	−0.81	1.74	−0.85	1.47	1.9	2.32

[a] Sample size = 16; total = 96.

Table 9. Statistical evaluation of 1985 study—determination of protein (%) by Hach Digesdahl instrumental and official AOAC methods

Product	Bias[a]	Std dev.
Hamburger	0.011	0.133
Pork sausage	0.094	0.211
Water-added ham	−0.197[b]	0.323[b]
Canned ham	−0.141[b]	0.244
Cooked sausage	0.094	0.125
Ground beef	−0.009	0.239

[a] Acceptable: bias ± 0.125; standard deviation 0.24.
[b] Unacceptable.

dahl) methods. The instrument shows promise for rapid protein determinations except for canned and water-added hams (Table 9).

An AOAC collaborative study will be conducted in 1986 by the Hach Co. to include calcium and phosphorus analyses.

Conclusion

With limited resources, FSIS must continue to adequately monitor the U.S. meat supply for composition and residues, so there is a definite need for rapid tests. USDA will continue to evaluate rapid meat compositional analysis techniques in regulatory situations, limiting most studies to those techniques that provide multiple tests. The tests and instruments for analysis of single analytes can and should be collaboratively studied through AOAC. Technical representatives from companies interested in such studies may contact the AOAC General Referee for Meat, Poultry, and Meat and Poultry Products. These representatives can be appointed as Associate Referees for specific rapid tests, to work with the General Referee and the methods committee statisticians to design a study to include all necessary categories of meat products. Such a study must define the method performance for within-laboratory and within-product standard deviations (S_O), standard deviations of the data (S_X), and the bias of the method compared to current official procedures. Components other than moisture, protein, fat, and salt should not be excluded from such studies. Instruments or techniques that have been improved recently, even if previously studied, can be reevaluated. Methods can be published complete with their performance characteristics and the user can decide whether the test meets the standards required for the particular analytical purpose.

PESTICIDE AND INDUSTRIAL CHEMICAL RESIDUES

Use of Solid Phase Florisil Cartridges to Separate Fat from Semivolatile Organic Compounds in Adipose Tissue

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A quick method for separation of semivolatile organic compounds from fat in adipose tissue has been developed. This method uses commercially available solid phase cartridges for sample cleanup. The results indicate that the recoveries, from hexane-extracted fat, of 4 representative classes of organic compounds range from 86.2 to 116%. The solid phase cartridges provide excellent separations of the fat from the analytes; no extraneous interference peaks were detected in the gas chromatograms. The method requires only 0.1 g sample and is quick and simple to use. Although results are reported for samples containing 1-14 ppm, the final extract can be concentrated to a volume allowing detection between 10 and 100 ppb.

The current method of the U.S. Environmental Protection Agency (EPA) to analyze adipose tissue for organic compounds calls for extraction with acetonitrile and partitioning of the analytes into petroleum ether by aqueous dilution of the acetonitrile extract (1). The concentrated petroleum ether fraction is then passed through a Florisil® column for further cleanup. Similar methods have been described in both the U.S. Food and Drug Administration's *Pesticide Analytical Manual* (2) and the AOAC *Official Methods of Analysis* (3). Even after these lengthy and involved procedures, some fatty residues remain in the sample extract. These have pronounced effects on the final analysis by gas chromatography (GC), both on the interpretation of the chromatograms and on the life span of GC columns and detectors.

A micro method for the determination of chlorinated pesticides in human and animal tissues is also included in the EPA *Manual* (4). The method uses only 0.5 g tissue. The compounds are extracted with hexane; cleanup and partitioning are conducted by successive elutions through a microcolumn of Florisil.

Gel permeation chromatography (GPC) has been proposed as the method of choice to clean up biological extracts, especially from high fat samples, for analysis to determine low molecular weight organic and pesticide residues. The National Human Adipose Tissue Survey (NHATS) is a major component of the National Human Monitoring Program which studies human exposure to toxic chemicals. The current proposed method (subject to multilaboratory validation) for this program uses GPC as well as Florisil for sample cleanup. However, the equipment itself is expensive, the procedure is labor intensive, and the method calls for the use of large quantities of a potentially hazardous solvent (methylene chloride).

In the present study, the feasibility of using commercially available cartridges to clean up adipose tissue extract was evaluated. Four organic compounds, each representing a different compound class, were used in the preliminary study. The results indicate that the recoveries from hexane-extracted fat of these 4 compounds, hexachlorobenzene (HCB), aldrin, polychlorinated biphenyls (PCBs), and polybrominated biphenyls (PBBs), range from 86.2 to 116%. The Florisil-type cartridge provides quantitative separation of the fat from the analytes, and there are no apparent extraneous interference peaks in the gas chromatograms obtained from samples after cartridge cleanup.

Received February 4, 1986. Accepted April 17, 1986.
Although the research described in this article has been funded by EPA (contract No. 68-03-3249), it has not been subject to the Agency's review and therefore does not necessarily reflect the views of the Agency and no official endorsement should be inferred.

Experimental

Reagents and Apparatus

(a) *Analytical standards.*—(EPA Pesticide Repository, Research Triangle Park, NC 27711). EPA code numbers: HCB, 3920; aldrin, 0080; PCB, 5705 (Aroclor 1254); PBB, P580; and 1,2-dichlorobenzene, 2280. All solvents were liquid chromatography or glass-distilled grade; chemicals were reagent grade from standard sources.

(b) *Preparation of primary and working standard solutions.*—*Primary standards*—Analytical standards were weighed to nearest 0.1 mg using electronic analytical balance, dissolved in hexane to final concentration of 100–200 μg/mL, and then stored in refrigerator. *Working standards*—Primary standards were further mixed and diluted to achieve appropriate peak heights in chromatogram. Between 0.1 and 1.4 μg of various standards were used for spiking 0.1 g hexane-extracted fat and subsequent loading onto cleanup cartridge.

(c) *Gas chromatograph.*—Hewlett-Packard Model 5880A equipped with ^{63}Ni electron capture detector and level-4 terminal recording integrator; capillary column, Durabond DB-5, 30 m × 0.25 mm id, 0.25 μm thickness (J&W Scientific, Rancho Cordova, CA 95670); split injector. Operating conditions: carrier gas, helium; temperatures—injector 285°C, detector 250°C, oven 60°C (2 min) then 10°/min to 310°C (10 min); injector volume, 2 μL; detector makeup gas, argon–methane (90 + 10).

(d) *Solid phase extraction column.*—Sep-Pak Florisil cartridges (Cat. No. 51960, 0.9 g packing material) (Waters Associates, Milford, MA 01757).

Preparation of Hexane-Extracted Fat from Bovine Adipose Tissue

Bovine adipose tissues (either from cows fed with various organic toxicants or from control cows) were obtained from fatty tissue taken at sacrifice. Adipose was homogenized (powderized) by blending tissue in high-speed blender in presence of liquid nitrogen and sodium sulfate. Subsample of 5 g was extracted in tapered glass tissue grinder with 20 mL hexane. Extraction was done by hand-grinding until no apparent solid fat was left. Virtually all fat appeared to be dissolved with exception of small amount of connective tissue which settled quickly at rest. Extract was transferred to

Table 1. Percent recoveries of HCB, aldrin, PCBs, and PBBs from solid phase cartridges

Compounds, μg	Analysis 1	2	3	4	5	6	Mean	SD
HCB, 0.1	112	119	106	105	113	102	109.5	6.28
Aldrin, 0.38	113	128	113	111	116	104	114	7.88
PCBs, 0.8	115	138	120	114	110	98	116	13.1
PBBs, 1.4	128	152	128	105	111	103	121	18.6

50 mL volumetric flask. Glass tissue grinder was rinsed with additional hexane and combined with original extract. Additional hexane was added to volumetric flask to fill to volume.

Procedure

A 1 mL aliquot of hexane-extracted fat was used for evaluation of sample cleanup with solid phase cartridges. Cartridges were conditioned by washing with 20 mL hexane immediately before use. Samples containing fat alone, mixed standards only, and fat spiked with mixed standards were loaded onto separate cartridges. Analytes were eluted from cartridges with at least 20 mL hexane. One mL internal standard (1,2-dichlorobenzene) was added to eluate, and final volume was adjusted to 25 mL with hexane. Compounds were analyzed and quantitated with gas chromatograph equipped with electron capture detector.

Results

Recoveries of HCB, Aldrin, PCBs, and PBBs from Cartridges

For the solid phase cartridge to be used to clean up adipose tissue samples, it is first necessary to demonstrate experimentally that the compounds of interest (analytes) can be fully recovered from the cartridge by elution with an appropriate solution; at the same time the elution should separate the analytes from the fat. A control, using the unspiked fat, indicated that no fat was eluted from the cartridge during sample loading and subsequent analyte elution. Table 1 shows the percent recoveries of the 4 analytes from the cartridge in the absence of adipose sample.

Spike Recoveries of HCB, Aldrin, PCBs, and PBBs in Bovine Adipose Tissue

To evaluate only the separation of the target analytes from the sample matrix (fat) by the solid phase cartridge, the analytes were added to the 1 mL sample extract rather than spiked into the sample before extraction. Table 2 shows the percent recoveries of these 4 compounds after they were spiked into the hexane-extracted adipose tissue, loaded onto the cartridge, and then eluted by hexane as described in *Experimental.*

As shown in Table 2, recoveries of all but HCB were above 100%. HCB is known to be quite volatile; this may explain why its recovery was considerably lower than that of the other 3 compounds.

Figure 1 shows representative chromatograms of the analyses. The chromatograms from sample extracts cleaned up by this procedure (lower panel) are essentially indistinguishable from the chromatograms obtained by direct injection of the mixed standards (upper panel). This indicates that any potentially interfering compounds in the sample extract were removed by the cleanup procedure. Figure 2 shows the unspiked but cleaned up adipose tissues; these chromatograms further confirm the above conclusion, i.e., no detectable extraneous peaks exist in the sample extract after this cleanup procedure.

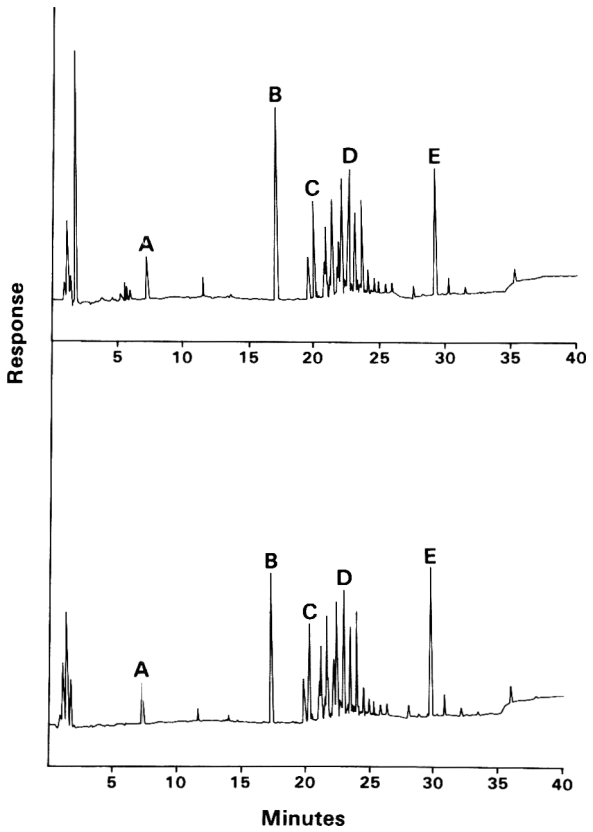

Figure 1. Chromatograms of mixed standards spiked into bovine adipose extract and separated from the fat by solid phase disposable cartridges (lower panel). Chromatograms of same mixed standards before spiking and subsequent cleanup (upper panel). A, 1,2-dichlorobenzene (internal standard); B, hexachlorobenzene; C, aldrin; D, polychlorinated biphenyls (Arochlor 1254); E, polybrominated biphenyls.

Discussion

The results obtained in this study indicate that a Florisil-type cartridge can be used as an alternative to GPC to clean up fatty samples such as adipose tissue. This procedure should be especially useful for quick screening of a large number of samples. Of the 4 classes of compounds tested, the recoveries were at least comparable with, if not better than, the conventional methods (unpublished data). In addition, the chromatograms from spiked samples were almost identical to those of pure standards, indicating that any interfering com-

Table 2. Percent recoveries of HCB, aldrin, PCBs, and PBBs spiked into bovine adipose extract

Compounds, μg	Analysis 1	2	3	4	5	6	7	Mean	SD
HCB, 0.1	90.3	88.4	84.4	84.7	92.2	78.4	85.1	86.2	4.57
Aldrin, 0.38	115	120	96.4	91.8	98.9	83.9	106	102	12.8
PCBs, 0.8	125	138	96.8	101	101	83.2	111	108	18.4
PBBs, 1.4	117	134	102	118	118	90.7	135	116	16.0

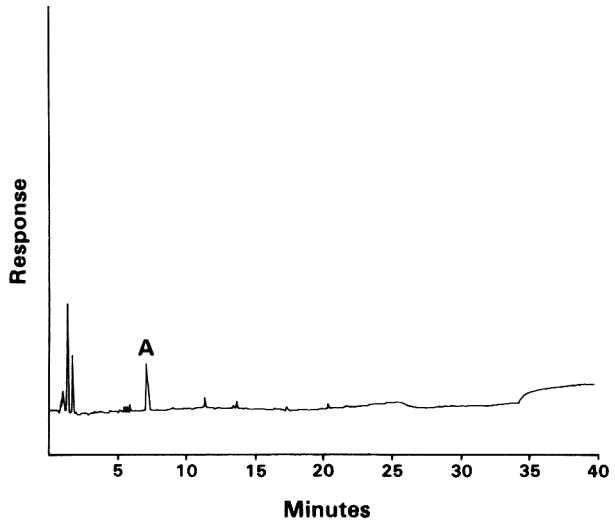

Figure 2. Chromatograms of unspiked adipose tissue cleaned up on solid phase disposable cartridges. A, 1,2-dichlorobenzene (internal standard).

pounds present in the sample extract were separated or eliminated from the analytes.

Since the major purpose of this study was to evaluate the use of a solid phase cartridge (Waters Sep-Pak) for cleanup of adipose tissue, the analytes were added to the hexane extract of the adipose tissue rather than spiked into the sample matrix before extraction. The extraction efficiency of each of these compounds has not been determined in this particular study, although numerous reports in the literature concern extraction of organic compounds from adipose tissue, including the Mills, Onley, Gaither method (5), the de Faubert Maunder method (6), and procedures in the EPA *Manual* (4). In addition, hexane has been used successfully for extraction of PCBs in human adipose tissue.

To eliminate any impurities inherent in the cartridges, preelution of the cartridges with at least 20 mL hexane is essential. It is also important to prevent the column from drying out throughout the procedure. Dry columns will interrupt the smooth flow of the elution; this may result in unacceptable performance of the cartridges such as low recoveries and poor reproducibility.

This cleanup procedure, as presented, provided an estimated detection limit between 1 and 14 ppm for the tested analytes, on the basis of using 0.1 g adipose tissue with a final sample volume of 25 mL. One should be able to improve the detection limit both by using larger quantities of sample and by reducing the final sample volume. Since each cartridge contains only about 0.9 g packing material, it may be difficult to substantially increase the sample size without overloading the cartridge. However, concentrating the final sample volume should not cause problems as long as the analytes are not highly volatile. A 100-fold reduction of the final sample volume (from 25 to 0.25 mL) could improve the detection limit to between 10 and 100 ppb for a capillary column and electron capture detector.

Even though bovine adipose was used for the development of this procedure, it is unlikely that human adipose would provide significantly different results. However, there is the possibility that human biopsy specimens would contain a larger proportion of fibrous or nonadipose tissues which may affect the recovery of certain analytes.

The slight upward shift of the baseline in the chromatogram from spiked samples may explain why some of the analytes (especially the late eluates such as PCBs and PBBs) show higher than 100% recovery. In addition, since both PCBs and PBBs are multicomponent compounds, it is possible that different congeners have different recoveries in this system.

Acknowledgments

The authors thank both G. A. Villa and M. E. Oakes for their help in preparing this manuscript. The feeding experiments were conducted by V. Bohman, University of Nevada–Reno. This study was performed at the Environmental Monitoring Systems Laboratory, Las Vegas, under contract No. 68-03-3249 with the U.S. Environmental Protection Agency.

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Extraction and Determination by Gas Chromatography of *S,S,S*-Tri-*n*-Butyl Phosphorotrithioate (DEF) in Fish and Water

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A simple, low-cost, fast method for the extraction and cleanup of DEF (*S,S,S*-tri-*n*-butyl phosphorotrithioate) from fish tissues and water samples was developed. The method combines extraction and cleanup in one step. The basis of the method is passing water samples or aqueous tissue homogenates containing DEF through a C-18 disposable cartridge. DEF is eluted from the cartridge by acetone or ethyl acetate. The eluates are analyzed by gas chromatography using a thermionic-specific detector. The method detects levels as low as 100 parts per trillion (ppt) in water samples; recovery efficiency from spiked fish tissues was greater than 95%. In addition, detectable levels of DEF were recovered from liver, brain, and muscle tissue of fish exposed to this compound. The method has a potential for use with other pesticides.

DEF (*S,S,S*-tri-*n*-butyl phosphorotrithioate) is commonly used as a cotton defoliant in California and the southeastern United States. In hens, DEF has produced delayed neurotoxic effects as well as acute cholinergic and hemotoxic effects (1–6). The specific toxic effect is dependent on the route of administration.

Recently, this organophosphorus compound has been shown to be toxic to fish at low concentrations under acute exposure (7). Under chronic exposure, DEF appears to adversely affect growth and survival of catfish and trout at levels below 10 ppb (8). DEF has a reported half-life in river water of 7 days, and traces persist for up to 8 weeks (9). Thus, due to widespread usage and potential for aquatic contamination, it has become important to develop rapid, sensitive analytical techniques.

Although analytical procedures exist for determining levels of DEF in produce, cottonseed, fish tissue, and water, these approaches are relatively time consuming and require fairly large samples (10–14). The procedures described below are simple, rapid, sensitive, and particularly useful when either small samples and/or many samples need to be analyzed.

Experimental

Reagents and Apparatus

(a) *Gas chromatograph.*—Varian Model Vista 6000 series, equipped with thermionic-specific detector (TSD). Temperatures—detector 300°C, injection port 250°C, isothermal column 230°C; gas flows—air 200 mL/min, hydrogen 4.5 mL/min, nitrogen carrier 23 mL/min.

(b) *Column.*—Glass, 2 m × 2 mm id, packed with 3% OV-17 on 80–100 mesh Chromosorb WP.

(c) *Solvents.*—ACS or liquid chromatography grade acetone, *n*-hexane, ethyl acetate, and methylene chloride.

(d) *DEF.*—Chromatography standard, 99.4% pure, provided by U.S. Environmental Protection Agency; 95% grade (Mobay Chemical Co., Kansas City, MO) was used for experimental work.

(e) *[^{14}C]DEF.*—Specific activity 23 mCi/mmole (Amersham Corp., Arlington Heights, IL).

(f) *Disposable cartridges.*—C-18 Sep-Pak (Waters Associates, Inc., Milford, MA).

(g) *Liquid scintillation spectrophotometer.*—Packard Model 3255 (Packard Instrument Co., Downers Grove, IL), with counts corrected for background and quenching. Duplicate samples counted for 10 min.

(h) *Disposable surgical syringes.*—3, 5, and 60 mL.

Animals

Juvenile blue channel catfish (*Ictalurus punctatus*), averaging 25–30 g wet weight, were used for tissue recovery studies. Some animals were sacrificed and liver tissue samples or whole-animal homogenates were spiked with 1 ppm [^{14}C]DEF and several concentrations of nonradioactive DEF. Other animals were exposed to 2.0 ppm nonradioactive DEF in 50 L aquariums; tissues of liver, brain, and tail musculature typically weighing between 0.2 and 0.5 g subsequently were analyzed.

Procedures

Water samples.—Water samples (100–500 mL) were spiked with nonradioactive DEF at concentrations ranging from 0.1 ppb to 6.0 ppm. After thorough mixing, duplicate 5–100 mL aliquots were loaded into disposable syringes, then passed through disposable cartridges at rate of approximately 2–3 mL/min. Each cartridge was air-dried by forcing air through syringe, then eluted with 3 successive 1 mL volumes of acetone, which were combined for chromatographic analysis. For water samples containing low levels of DEF, ethyl acetate replaced acetone for eluting the cartridges. The combined eluate was evaporated to dryness under a gentle stream of nitrogen, and the residue was redissolved in a smaller volume of acetone. A 2 μL aliquot of the final acetone extract was injected into the gas chromatograph for analysis. The method was originally developed by tracing [^{14}C]DEF in a water sample spiked to 8 ppm.

Tissue samples.—Tissue samples were homogenized in a 0.05M phosphate buffer, pH 7.4, to produce 10% w/v homogenate. Two methods for extracting DEF from tissue samples were tested, and efficiency of 3 solvents (*n*-hexane, ethyl

Table 1. Recovery of DEF from spiked water samples[a]

Concn	Eluting solvent	Sample volume, mL	Recovery, % (mean ± SD)
6.0 ppm	acetone	5	106 ± 4.9 (4)[b]
3.0 ppm	acetone	5	95.9 ± 3.2 (4)
1.0 ppm	acetone	10	99.6 ± 3.3 (3)
600 ppb	acetone	10	102 ± 4.3 (3)
100 ppb	ethyl acetate	50	101.2 ± 3.1 (3)
10 ppb	ethyl acetate	50	105.5 ± 6.6 (4)
1 ppb	ethyl acetate	100	109.0 ± 7.6 (4)
100 ppt	ethyl acetate	100	113.0 ± 9.3 (4)

[a] Samples (5–100 mL) passed through a cartridge, which was eluted with 3 successive 1 mL volumes of acetone or ethyl acetate, then analyzed by GC. Analytical conditions are as described in Figure 1.
[b] Numbers in parentheses represent the number of determinations.

Received December 9, 1985. Accepted April 10, 1986.
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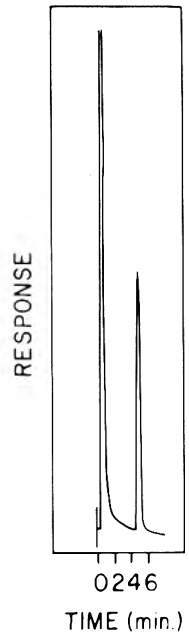

Figure 1. Gas chromatogram of 1 ng DEF standard on 2 m, 3% OV-17 glass column. System used: isothermal at (C°), 230, 250, and 300 for the column, injector, and TSD detector, respectively. Flow rate (mL/min) air, 200; H_2, 4.5; and N_2, 23.

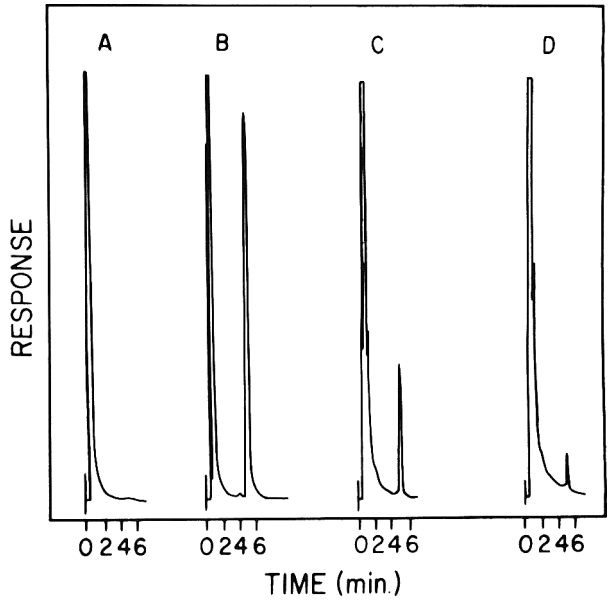

Figure 2. Gas chromatograms of water samples spiked with DEF and extracted using cartridges and ethyl acetate as eluting solvent. A, 50 mL control; B, 10 mL sample at 1 ppm; C, 100 mL sample at 1 ppb; D, 100 mL sample at 100 ppt. Analytical conditions as described in Figure 1.

acetate, and methylene chloride) was compared. In the first method, an aliquot of tissue homogenate (typically 0.5 or 1.0 mL) was thoroughly mixed with 4 volumes of solvent for 1 min. Samples were centrifuged to separate aqueous and organic layers. The organic layer was saved, and the aqueous phase was reextracted twice; all organic layers were combined for analysis. If emulsions developed, samples were allowed to stand and were recentrifuged as needed.

In the second method, we passed tissue samples through the C-18 cartridges. For this method, a small sample (0.2–1 mL) was diluted 20-fold with deionized water, loaded into a syringe, and passed slowly through the cartridge. The cartridge was then eluted with 5 successive 1 mL volumes of organic solvent. Solvent extracts were combined and concentrated for gas chromatographic analysis. For radiotracing work, solvent extracts were not combined; rather, the amount of DEF removed with each successive elution was determined.

Table 2. Comparison of tissue extraction methods and solvents[a]

	Cumulative recovery from successive elutions of [^{14}C]DEF[b], %					
	Liver homogenate extracts			Whole-fish homogenate extracts		
Solvent	1	2	3	1	2	3
Solvent method						
Hexane	56.7	75.7	81.6	58.2	80.6	86.6
Ethyl acetate	44.4	84.8	94.8	77.3	94.7	99.1
Methylene chloride	11.8	21.2	34.1	44.4	73	82.7
Cartridge method						
Hexane	26.2	40.4	48.6	19.6	38.2	49
Ethyl acetate	70.1	91.2	97.3	88.5	99.1	103.7
Methylene chloride	64.4	89	95.7	92.1	106	110.9

[a] Tissues were spiked with [^{14}C]DEF and extracted by the solvent or cartridge method as described under *Experimental*.

[b] Aliquots from successive elutions were counted in the liquid scintillation counter. For the cartridge method, the 1st and 2nd 1 mL elutions were combined before counting, as were the 3rd and 4th elutions. See text for details.

Results and Discussion

Water Samples

Using [^{14}C]-labeled DEF, we determined that the cartridge retained virtually all of the DEF contained in water samples passed through it, with the water filtrate having counts just slightly above background. The first 1 mL volume of acetone eluted approximately 50% of the DEF that had been retained by the cartridge; two 1 mL volumes eluted 90%; 3 volumes of acetone eluted over 98%. Confirmation of this extraction efficiency was provided by gas chromatographic analysis of water samples spiked with 95% nonradioactive DEF (Table 1). A typical chromatogram of 1 ng DEF is shown in Figure 1. Under isothermal column conditions, the retention time for DEF was 5 ± 0.1 (mean ± SD) min. Peak heights of DEF were linear between 0.2 and 7 ng, and amounts as low as 25 pg were detected. The principle of concentrating samples under a nitrogen stream permits a wide range of ambient

Table 3. Recovery of DEF from spiked tissue samples by gas chromatographic analysis[a]

Concn	Sample volume, mL	Recovery, % (mean ± SD)
Liver		
1 ppm	0.5	90.1 ± 5.8 (4)[b]
100 ppb	0.5	90.0 ± 3.5 (4)
10 ppb	1.0	104.1 ± 2.8 (3)
Whole fish		
1 ppm	0.5	96.7 ± 5.4 (4)
100 ppb	0.5	98.1 ± 6.4 (5)
10 ppb	1.0	111.5 ± 6.0 (4)

[a] Aliquots of homogenates (0.25–1 mL) diluted 20-fold with water, then passed through cartridge, which was eluted with 5 successive 1 mL volumes of ethyl acetate, concentrated, and analyzed by GC. Analytical conditions are as described in Figure 1.

[b] Numbers in parentheses represent the number of determinations.

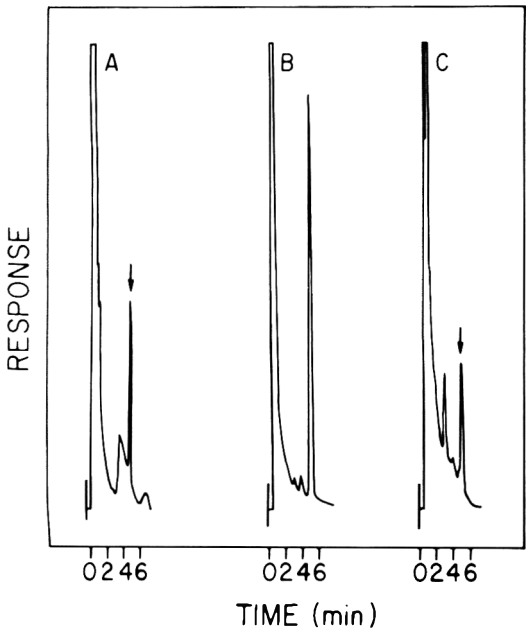

Figure 3. Gas chromatograms of extracts of tissue homogenates spiked with DEF, extracted using cartridges and ethyl acetate as eluting solvent. See text for details. A, 250 μL aliquot of liver homogenate at 1 ppm; B, 500 μL aliquot of whole-fish homogenate at 1 ppm; C, 1 mL aliquot whole fish homogenate at 10 ppb. Arrows indicate DEF peak. Analytical conditions are as described in Figure 1.

values to be detected, including amounts as low as 100 ppt (Figure 2).

Tissue Samples

Liver and whole-body homogenates were each extracted, testing 3 solvents and 2 methods. A tissue sample was spiked to 1 ppm (wet weight) with radioactive DEF and homogenized in phosphate buffer. Before extraction, homogenates were thoroughly mixed, then duplicate aliquots were tested. The cumulative recovery of [^{14}C]DEF in successive extractions for each of the 3 solvents tested is given in Table 2. Briefly, using the solvent extraction method, *n*-hexane resulted in approximately 82% recovery of [^{14}C]DEF, ethyl acetate, nearly 95%, while methylene chloride resulted in only about 35% recovery for liver tissue. Results were similar, but slightly better, using the same method on samples of whole-body homogenate, except in the case of methylene chloride, which yielded approximately 83% recovery. This difference in recovery between the 2 homogenates is probably partially attributable to the thoroughness of mixing the solvent and the homogenate, because methylene chloride seemed to be more difficult to mix well with the liver homogenate. Using the disposable cartridge method, recovery with hexane as the eluting solvent was relatively poor for both tissues (about 50%). For both ethyl acetate and methylene chloride, the cartridges yielded over 95% recovery of [^{14}C]DEF. The recovery, as determined by gas chromatographic analysis, of nonradioactive DEF from tissue homogenates spiked with 10 ppb to 1 ppm is shown in Table 3. Examples of tissue extracts analyzed by GC are shown in Figure 3.

In addition to testing the homogenates mentioned above, several fish were placed in water containing 2.0 ppm DEF for 24 to 96 h. Liver, brain, and muscle tissues were then excised, homogenized, and extracted with ethyl acetate. Considerable amounts of DEF were detected in each tissue under conditions resembling experimental conditions that are commonly used (Figure 4).

Extraction Efficiency

One important aspect of sample retention on the cartridge appears to be the speed at which the sample and solvent are passed through the cartridge, with slower speeds (3 mL/min or less) improving recovery.

Eluting the cartridge with one 5 mL volume of acetone did not remove DEF from the cartridge as effectively as did several successive volumes of acetone. Also, the cartridges are reusable if cleaned with several volumes of acetone between samples. These cartridges have been used over a dozen

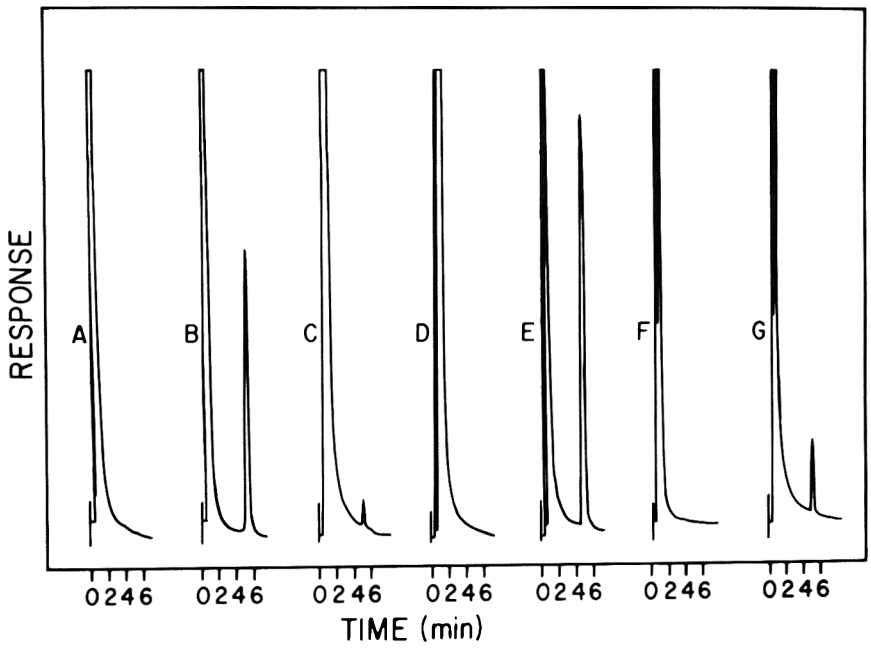

Figure 4. Gas chromatograms of extracts of tissues excised from fish exposed to 2.0 ppm DEF. Tissues were extracted using cartridges and ethyl acetate as eluting solvent. See text for details. A, brain from control fish; B, brain from fish exposed for 48 h; C, brain from fish 21 days after a 96 h exposure; D, liver from control fish; E, liver from fish exposed for 24 h; F, tail musculature from control fish; G, tail musculature from fish exposed for 48 h. Analytical conditions are as described in Figure 1.

times with no discernible loss in their ability to retain DEF from water samples. For tissue samples, the cartridges will eventually become clogged. Backflushing with a mixture of solvents, such as methylene chloride followed by acetone, can extend the life of the cartridge, and depending on the tissue type, a cartridge normally lasts for 6 to 10 samples.

In general, samples extracted using the cartridges were cleaner than those extracted with the solvent method, and no problems were encountered with fatty substances contaminating either the column or the detector in the course of approximately 300 tissue analyses. The column was baked periodically during these analyses. Methylene chloride extracts obtained by the solvent method contained considerable amounts of contaminating substances, so the use of this solvent was discontinued. Both hexane and ethyl acetate extracts were relatively clean, and although occasional additional peaks occurred, these peaks did not coincide with or obscure the DEF peak (Figure 3). Also, as others have noted (11, 14), the specificity of the TSD detector minimizes the necessity of additional cleanup procedures for organophosphorus compounds. Given the good recovery and relatively clean chromatogram, ethyl acetate appears to be a very suitable solvent. Ethyl acetate has also been used for eluting water samples and may be preferable to acetone for samples that require concentrating, since water can be removed.

Recovery of DEF from fish tissue and water samples by using Sep-Pak cartridges (95%) compares quite favorably with the 90–95% recoveries reported for the AOAC multiresidue and methylene chloride extraction procedures (10–12). Coupled with this good recovery is the capability of detecting lower levels (100 ppt) in water than have been reported for any of the other methods. The high sensitivity makes this method particularly useful for analyzing residues in small organisms, or specific organs of an animal, since small amounts of homogenates are sufficient. This technique could also be used in conjunction with gas chromatography/mass spectroscopy or additional columns for unequivocal identification of unknown field samples.

Another asset of this procedure is its simplicity, and concomitant reduction in sample handling time, particularly for tissue samples. In addition, costly special equipment is not required, and the amounts of organic solvents used are relatively small.

In summary, the cartridge extraction procedure combines high sensitivity and good recovery with a methodology that is relatively fast, simple, and inexpensive. This method may be used for the extraction and analysis of other pesticides in fish and water.

Acknowledgment

This work was supported in part through the Duke University Marine Laboratory Visiting Scholar program and the National Toxicology Training program, grant number 32ES 07031.

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On-Column Partition Cleanup of Fatty Extracts for Organophosphate Pesticide Residue Determination

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A fast, single-step, and efficient partition between *n*-hexane and acetonitrile on ready-to-use, disposable mini-columns of Kieselghur-type material has been developed for the cleanup of fatty extracts for organophosphate (OP) pesticide residue determination by gas chromatography with flame photometric detection. Nine OP pesticides (diazinon, etrimfos, chlorpyrifos-methyl, pyrimiphos-methyl, chlorpyrifos, bromophos, bromophos-ethyl, malathion, fenitrothion) most commonly used for protection of stored cereals, oil seeds, and legumes were separated from up to 2.0 g lipidic material with recoveries between 80 and 107% at spiking levels ranging for the different compounds from 0.1 to 5.0 ppm.

Received December 9, 1985. Accepted April 8, 1986.

One of the major problems the analyst faces in the determination of organophosphate (OP) pesticides in fatty foodstuffs such as cereals, oil seeds, and legumes is the isolation of pesticide residues from the bulk of lipidic coextractives. In fact, extracts from such commodities, obtained with general extraction procedures (1–6) or even with procedures specifically designed for grain (7–10), contain considerable amounts of coextractives. In these cases, direct determination by gas chromatography (GC) (5, 6) is impractical even with selective detectors such as the flame photometric detector (FPD) or alkali flame ionization detector (AFID). For the isolation of pesticide residues from lipidic coextractives,

Table 1. Amounts of lipidic material released into eluate compared to the amount applied to disposable column for different foodstuffs

Foodstuff (g)	Lipid applied to column, mg	Lipid in eluate, mg
Olive oil	1.00 g	17–24[a]
	1.50 g	23–27[a]
	2.00 g	29–32[a]
Wheat (10.00)	135	21
Maize (10.00)	410	35
Barley (10.00)	125	10
Rice (10.00)	125	43
Peanuts (4.00)	1080	10
Soya beans (10.00)	1420	40
Beans (10.00)	225	15
Chick peas (10.00)	500	27
Lentils (10.00)	135	17

[a] Range for 6 determinations.

several techniques can be used prior to further cleanup and/or final determination by GC with flame detectors.

Due to the wide range of polarity of OP pesticides, adsorption chromatography may not be useful as a general, broad-spectrum cleanup system. Separatory funnel partitioning techniques between immiscible solvents, for instance, petroleum ether–acetonitrile (1, 2), *n*-hexane–dimethylformamide (11), petroleum ether–dimethylsulfoxide (12), are time-consuming and require a back-transfer into low-boiling, low-polarity solvents and, consequently, much glassware. Furthermore, troublesome emulsions frequently occur (2, 12) especially with lipidic extracts from oil seeds. In such cases, partition chromatography (13, 14) is a useful alternative method. However, both these methods have some drawbacks, e.g., back-transfer into low-boiling solvents if water–acetonitrile is the eluting solvent (13), column preparation and/or conditioning prior to use (13, 14), and applicability limited to pesticides with extraction *p*-values (fraction of solute partitioning into nonpolar phase of an equi-volume 2-phase system) (15) of ≤0.05 (14). Gel permeation chromatography (GPC), with different matrix–eluant combinations (16–19), is also used. Although GPC may be automated, it is lengthy and a considerable volume of solvents is required. Sweep codistillation (2, 20, 21) is better suited to OP residues in nonfatty foodstuffs, and some decomposition of critical chemicals can occur.

The need for conducting a survey of OP pesticide residues in stored cereals, oil seeds, and legumes prompted us to seek a simple, straightforward, broad-spectrum system for isolation of pesticide residues from the bulk of lipidic coextractives. We found that the petroleum ether–acetonitrile partitioning (1, 2) can be carried out advantageously on disposable, ready-to-use columns filled with Extrelut®, a macroporous Kieselghur-type material. The system offers a fast, single-step partition cleanup which effectively isolates 9 OP pesticide residues having a wide range of extraction *p*-values from lipidic coextractives. The resulting extracts are directly amenable to gas chromatographic determination with a flame photometric detector operated in the phosphorus mode (GC/FPD-P).

METHOD

Apparatus and Reagents

(a) *Gas chromatograph.*—Perkin-Elmer, Model Sigma 4B, or equivalent, with FPD operated in phosphorus mode.

(b) *GC column.*—1.8 m × 4 mm id, Pyrex glass, packed with 5% QF-1 on 100–120 mesh Chromosorb W-HP. Operating conditions: helium carrier gas 60 mL/min; column oven 190°C; inlet and outlet blocks 225°C.

(c) *Ready-to-use columns.*—Extrelut-3, Cat. No. 15327 (E. Merck). Fix needle (Luer-Lock 0.65/32) at column end as flow regulator.

(d) *Reference standards.*—(Compounds of analytical purity from the collection of this laboratory.) Prepare in acetone as follows: 1 μg/mL diazinon, 2 μg/mL etrimfos, 2 μg/mL chlorpyrifos-methyl, 1.5 μg/mL pyrimiphos-methyl, 2.5 μg/mL chlorpyrifos, 3 μg/mL bromophos, 3 μg/mL bromophos-ethyl, 5 μg/mL malathion, 3 μg/mL fenitrothion.

(e) *Solvents.*—*n*-Hexane, acetonitrile (saturated with *n*-hexane), acetone, methanol (all redistilled in glass).

Table 2. Range of recovery values of 9 organophosphate pesticides from 1.00, 1.50, or 2.00 g olive oil spiked at different levels

Pesticide	*p*-Value[a] This lab.[b]	*p*-Value[a] Others	Spiking levels, ppm	Recoveries, %
Diazinon	0.28	0.28[c]	0.10–1.0	84–98
Etrimfos	0.20	—	0.20–2.0	83–100
Chlorpyrifos-methyl	0.18	0.17[d]	0.20–2.0	90–98
Pyrimiphos-methyl	0.21	—	0.15–1.5	81–102
Chlorpyrifos	0.27	0.26[d]	0.25–2.5	84–97
Bromophos	0.26	—	0.30–3.0	84–99
Bromophos-ethyl	0.43	—	0.30–3.0	80–88
Malathion	0.05	0.042[c]	0.50–5.0	90–107
Fenithrothion	0.06	0.03[d]	0.30–3.0	86–100

[a] *p*-Values between *n*-hexane and acetonitrile.
[b] *p*-Values by GC/FPD-P after single distribution.
[c] *p*-Values of Bowman and Beroza (15).
[d] *p*-Values of Dale and Miles (14).

Procedure

In graduated test tube, weigh up to 4.00 g oil or lipidic material resulting from extraction of oily crops with one of the general procedures (1–6). Dissolve and dilute to 6 mL with *n*-hexane.

Pipet 3 mL of above lipid solution onto top of disposable column. Let solution drain into filling material. Wait 10 min to obtain even distribution. Then elute column with three 5 mL portions of acetonitrile equilibrated with *n*-hexane. Collect eluate in 50 mL Erlenmeyer flask. Add 4 mL methanol. Concentrate eluate to dryness by means of rotary evaporator (50–55°C water bath, reduced pressure). Remove last traces of solvent with gentle stream of nitrogen. Add appropriate volume of acetone (1–2 mL) and inject 1–5 μL into gas chromatograph. Calculate amount of OP pesticides by comparing peak heights of unknown samples with equivalent peaks of standards according to well-known procedures.

Partitioning Column Performance

Pipet 0.4, 1.0, or 2.0 mL standard solution into 10 mL graduated test tubes (use 3 tubes for each spiking level). Evaporate solvent by rotating tube while holding in horizontal position. Weigh 2.00, 3.00, or 4.00 g refined vegetable oil, respectively, into tubes of each spiking level. Dilute to 6 mL with *n*-hexane. Follow above procedure, starting from "Pipet 3 mL . . .", but collect eluate in weighed flask and determine amount of lipidic material released from column. Determine percent recoveries of OP pesticides by GC/FPD-P.

Results and Discussion

By applying a total volume (lipid + solvent) of 3 mL, only minimal amounts of lipids were released into the eluate when

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up to 2.00 g portions of olive oil, or different amounts of lipids from oily crops, were partitioned on the column (see Table 1). Under the conditions adopted, the amount of lipids released into the eluate did not vary greatly with the amount applied. Small variations could also be attributed, for instance, to the type of lipidic material, to the temperature at the partitioning time, and so on. The lipid removal was only slightly worse than that reported by Dale and Miles (14) for partition chromatography on Florisil, but definitely better than that obtainable in separatory funnel partitioning techniques (as much as 200 mg or more of lipids unresolved for a sample of 2.0 g subjected to partition) (1, 2).

Pesticide recovery was investigated for the 9 OP pesticides in this study, which are the most commonly used for protection of stored cereals, oil seeds, and legumes. An efficient separation of these pesticides was achieved with a GC column containing 5% QF-1 on Chromosorb W-HP. The eluates from the partitioning column were analyzed by GC/FPD-P without further cleanup. Should a further cleanup be necessary, e.g., for confirmation, minicolumn chromatography or even thin layer chromatography can be used to remove the minor amounts of lipids released into the eluates. The gas chromatograms were all free from interfering peaks and were indistinguishable from those obtained with the standard solution of pure pesticides. Furthermore, due to the low amount of lipids injected (10–40 μg coextractives per 1 μL injection), the injector port of the gas chromatograph could afford many injections of sample extracts before cleaning was necessary, and the column did not display any serious loss of performance. For the recovery experiments, blank olive oil was used as a model lipidic matrix. To test a possible effect of the lipid load, the recovery of pesticides was determined (in triplicate) at 9 different spiking levels, with 3 spiking levels associated to each level (1.00, 1.50, or 2.00 g) of oil load. At the levels tested, ranging for the different compounds from 0.1 to 5 ppm, recoveries were always greater than 80% for all compounds, approaching complete recovery in most cases. The results of the recovery experiments with olive oil are summarized in Table 2. The recoveries were not related to either the spiking level or the lipid load in the ranges tested. Eluting the columns with further 10 mL acetonitrile recovered an average of 3–6% more of the different pesticides. However, especially in the screening of a large number of samples, the conditions selected appear to be the best compromise between recovery and time/cost of analysis.

Recovery experiments were also carried out on the crops listed in Table 1 fortified with the standard compounds at the lowest levels used for olive oil. The crops were extracted according to ref. 3, and the raw lipidic extract was cleaned up according to the present method. The recoveries of the pesticides, including the extraction, were in the range 90–97% for all the crops tested.

The extraction p-values of the compounds studied can be used as a criterion to estimate the range of applicability of this cleanup. As can be seen from the extraction p-values given in Table 2, pesticides having p-values between n-hexane and acetonitrile of ≤ 0.43 were satisfactorily recovered from up to 2.00 g olive oil. That means a wider applicability of this method compared with the method of Dale and Miles (14) which is limited to pesticides with p-values of ≤ 0.05. Furthermore, if one considers that only 12 of 138 pesticides and related compounds investigated by Bowman and Beroza (15) and Beroza et al. (22) have p-values between n-hexane and acetonitrile of > 0.43, the potential of the present method for the cleanup of pesticides and other environmental pollutants in fatty matrixes is apparent.

In conclusion, the main features of this cleanup system compared with the above-quoted methods are the good cleanup and recoveries, the minimum of glassware and reagents required, the lack of emulsions, the reduced time for a single cleanup (15 min), and the simplicity of the operations involved that allows several samples to be run in rapid sequence.

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Automated Sample Cleanup for Pesticide Multiresidue Analysis. III. Evaluation of Complete System for Screening Subtolerance Residues in Vegetables

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An automated continuous flow sample cleanup system intended for rapid screening of foods for pesticide residues in fresh and processed vegetables has been developed. Recovery and precision data for 8 pesticides in each of 3 crops are compared for the automated and manual procedures. Average recovery for samples fortified with pesticides between 0.026 and 0.277 ppm was 98% for the automated system and 92% for the manual procedure. Average coefficient of variation was 6.6% for the automated system and 4.2% for the manual procedure. In another evaluation, the automated system gave an average recovery of 95% for 12 pesticides commonly found in imported foods; the manual procedure gave an average recovery of 91%. Thus, the results obtained so far indicate that the automated system for sample cleanup gives results comparable to those obtained by manual procedures.

The determination of pesticide residues in foods of unknown spray history is a formidable task, because it involves the identification and quantitation of several hundred possible single compounds or combinations in the presence of complex matrixes. The conventional analytical methods commonly used are time-consuming, labor-intensive, and costly in terms of expensive solvents and absorbents. This is because the pesticides, which are present at parts per million (ppm) levels, have to be separated from the food matrix (sample cleanup) so that they can be identified and measured by gas or liquid chromatography. Automated systems are commercially available for the chromatography steps but not for the cleanup steps. We have developed a system intended for rapid screening of crop samples. This system consists of 2 parts, a solvent partitioning module and a column chromatography module. Both of these systems have been evaluated separately (1, 2) for precision and accuracy. We now provide data for the evaluation of the integrated system.

Experimental

Instrumentation

A block diagram of the system is given in Figure 1. Detailed descriptions of the design and operation of the individual modules were published earlier (1, 2).

The solvent partitioning module (SPM) is interfaced to the column chromatography module (CCM) so that the sample extract in hexane, after leaving the third phase separator of the SPM, is automatically pumped to port 3 of the CCM 10-port sampling valve.

Operation of the System

To start processing samples, the reservoirs are filled with their appropriate solvents or solutions and the proportioning pump of the SPM is turned on. The SPM is allowed to equilibrate for 30 min while the sample line is primed with 35% water–acetonitrile (350 mL water diluted to 1 L with acetonitrile). Florisil columns are packed and connected to the 60-port switching valve of the CCM. The fraction collector is loaded with empty tubes and power is applied to the remaining system components.

Filtered crop extracts (1) in acetonitrile are loaded in place and the first cycle is initiated by activating the sampler. The system has been designed so that it takes approximately 10 min for the extract containing the compounds of interest to pass through the SPM and arrive at the 10-port sampling valve. At an elapsed time of 9 min, the CCM is started. This timing provides a 1 min interval to allow for any minor fluctuations in component synchronization. The extracts are cleaned up by the CCM and the column eluates are collected. The first sample is completed in 90 min and each additional sample requires 20 min. Further details pertaining to system operation were presented previously (1, 2).

Evaluation of System

Five replicate samples, 100 g each, of homogenized tomatoes, squash, and green beans were analyzed. Each sample was weighed into a high-speed blender jar, and 198 mL acetonitrile and 2 mL of a spiking solution containing 8 pesticides were added. This spiking solution was prepared in acetonitrile and contained, per mL, 1.225, 2.853, 2.134, 2.913, 12.88, 8.122, 11.88, and 3.83 μg lindane, ronnel, aldrin, heptachlor epoxide, ethion, *p,p'*-DDT, parathion, and dieldrin, respectively. After blending at high speed for 2 min, the contents were filtered with suction through a 12 cm Buchner funnel (Whatman No. 1 paper) into a 500 mL filter flask. The filtrate was transferred to a 250 mL graduated cylinder and the volume was recorded. A 50 mL aliquot was transferred to a sample tube and loaded in the automated sampler tray. The automated sample cleanup was started after all 15 samples had been thus prepared.

The remaining filtrates (ca 200 mL) were each cleaned up by a standard manual procedure (3). Analyses were performed on a Tracor 550 gas chromatograph equipped with electron capture and flame photometric detectors and 1.8 m × 2 mm id glass columns packed with 5% OV-101 on 80–100 mesh Chromosorb WHP. Carrier gas (nitrogen) flow rates were 30 mL/min. Column temperature (ca 200°C) was adjusted to permit elution of *p,p'*-DDT at 3.13 relative to chlorpyriphos; detector sensitivity was adjusted to provide ½ FSD for 1.5 ng chlorpyriphos.

Identical procedures were used for the cleanup and analysis of cucumbers spiked to contain 0.072 ppm chlorpyriphos, 0.095 ppm diazinon, and 0.091 ppm nitrofen; red peppers spiked to contain 0.023 ppm quintozene (PCNB), 0.063 ppm vinclozolin, and 0.108 ppb methyl parathion; and eggplant spiked to contain 0.048 ppm dacthal, 0.329 ppm *p,p'*-methoxychlor, and 0.270 ppm ethion.

Two 25 g samples of dried beans were each spiked with 2 mL acetonitrile solution containing, per mL, 2.505, 2.188, and 4.930 μg lindane, heptachlor, and parathion, respectively, in a blender jar. To this was added 348 mL water–acetonitrile (35 + 65) solution.

Received August 13, 1985. Accepted May 19, 1986.
Presented in part at the Institute of Food Technologists National Meeting, June 9–12, 1985, Atlanta, GA. This research was supported by the Food and Drug Administration Science Advisor Research Associates Program (Project No. 10539), by the State of New Jersey, and by the U.S. Department of Agriculture (Regional NE-83). New Jersey Agricultural Experiment Station Publication No. D-10201-3-85.

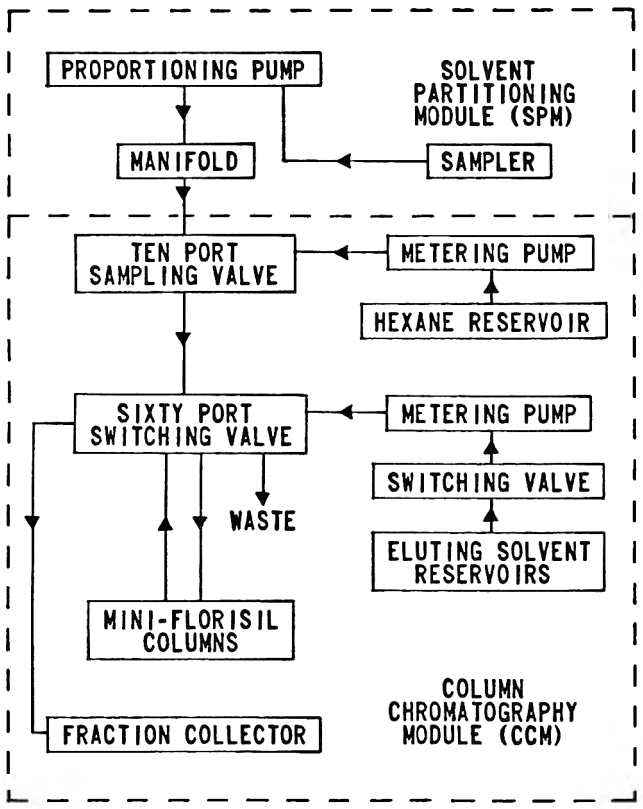

Figure 1. Block diagram of complete continuous flow system.

Calculation of Equivalent Sample Weight

Calculations for the automated system are based on the AOAC method, sec. **29.011(f)**, where equivalent sample weight is given as $S \times (F/T) \times P/100$; S = g sample taken, F = mL filtrate, T = total volume (mL water in sample + mL CH_3CN added − correction in mL for volume contraction), P = mL hexane extract, and 100 = mL hexane into which residues were partitioned. For our calculations, S and T are as defined above. However, P/100 = percent hexane recovered by solvent partitioning module and F = volume (mL filtrate aspirated by sample in 16 min). Data presented in this report (see *Results and Discussion*) are based on experimentally obtained values of F = 40 mL and $P/100$ = 0.85 for all the high moisture products (tomatoes, green beans, squash, cucumbers, red peppers, and eggplants). For dried beans, F = 40 mL; $P/100$ = 0.70.

Results and Discussion

The system we have developed is intended for use as a rapid screening procedure for monitoring pesticide residues in vegetables. Since approximately 95% of the fresh and processed vegetables analyzed by the Food and Drug Administration during the past 10 years contained either no residues or residues below established tolerances, these procedures should work well most of the time. We have, therefore, chosen sample fortification levels most frequently encountered in our experience, i.e., below tolerance levels, to evaluate the system. In actual practice, the presence of a violative residue might cause the residue to be carried over to subsequent samples; the number of samples depends on the level of contamination in the violative sample. Such a situation could be remedied by increasing the wash time cycle, thus increasing the overall analysis time. An approach more consistent with decreasing overall analysis time would be to reanalyze violative samples by the manual method.

Recovery and precision data for 8 pesticides in 3 different crops are presented in Table 1. Fortified tomato samples gave recoveries of 81–107% (average 92%) for the automated system and 68–106% (average 88%) for the manual procedure. Coefficients of variation (CVs) were 2.4–13.0% (average 6.5%) and 2.4–9.7% (average 5.2%) for the automated and manual techniques, respectively. The same pesticides were recovered from green beans at 92–109% (average 99%) for the automated method and 83–111% (average 97%) for the manual method. The CVs were 3.3–8.1% (average 5.3%) for the automated technique and 0.2–3.5% (average 2.4%) for the manual procedure. Analysis of squash gave average recoveries of 90–111% (average 99%) with CVs of 6.3–9.5% (average 7.7%) for the automated system. The manual procedure gave average recoveries of 75–106% (average 90%) and CVs of 3.3–7.7% (average 5.1%). In general, the automated cleanup system exhibited excellent recoveries of the 8 representative pesticides from the 3 crop substrates with somewhat higher CVs than those by the manual method.

Table 1. Comparison of automated and manual cleanup procedures for analysis of 8 pesticides in tomatoes, green beans, and squash

	Automated[b]			Manual[b]		
Pesticide[a]	Found, ng/g	Rec., %	CV %[c]	Found, ng/g	Rec., %	CV %[c]
Tomatoes						
Lindane	26	107	7.5	24	99	3.4
Ronnel	54	94	4.5	52	91	6.9
Aldrin	37	87	13.0	45	106	4.2
Heptachlor epoxide	53	92	6.2	52	89	6.2
Ethion	209	81	6.9	175	68	9.7
p,p'-DDT	145	87	5.8	138	85	5.0
Parathion	236	99	5.7	210	88	3.5
Dieldrin	69	90	2.4	60	78	2.4
Green beans						
Lindane	27	109	4.4	27	109	2.4
Ronnel	58	102	5.2	56	99	3.2
Aldrin	40	93	6.8	47	111	1.7
Heptachlor epoxide	58	100	8.1	57	98	2.6
Ethion	236	92	3.3	213	83	3.5
p,p'-DDT	157	97	5.0	162	100	3.0
Parathion	237	100	4.3	198	83	0.2
Dieldrin	75	98	5.0	72	94	2.3
Squash						
Lindane	28	111	7.3	24	99	7.7
Ronnel	58	102	6.3	50	87	5.3
Aldrin	43	102	9.5	45	106	5.6
Heptachlor epoxide	60	103	8.2	49	85	4.2
Ethion	233	90	6.5	194	75	6.1
p,p'-DDT	160	98	6.7	149	92	4.2
Parathion	277	96	8.5	204	86	4.4
Dieldrin	71	93	8.3	70	91	3.3

[a] CAS Registry numbers: lindane, 58-89-9; ronnel, 299-84-3; aldrin, 309-00-2; heptachlor epoxide, 1024-57-3; ethion, 563-12-2; *p,p'*-DDT, 50-29-3; parathion, 56-38-2; dieldrin, 60-57-1.
[b] Five replicates.
[c] Percent coefficient of variation.

In 3 collaborative studies performed (4–6) using the method of Mills et al. (7) a total of 9 pesticides were evaluated at concentrations of 0.05–10 ppm. The composite CV from the 3 studies was 15% (8). The results of 10 collaborative studies of the manual procedure for nonfatty foods were evaluated by Burke (9). A total of 18 organochlorine and organophosphorus pesticides covering the concentration range 0.03–17 ppm in 121 pesticide/commodity combinations were ana-

Table 2. Comparison of automated and manual cleanup procedures for various pesticides in cucumbers, red peppers, eggplants, and dried beans

Pesticide[a]	Spiking level, ng/g	Manual rec., %	Automated rec., %	GC detector	Commodity
Chlorpyrifos	72	81,91	75,81	EC	cucumbers
Diazinon	95	86,101	98.116	FPD	cucumbers
Nitrofen	91	82,89	78,97	EC	cucumbers
Quintozene	23	95,106	106,121	EC	red peppers
Vinclozolin	63	93,117	111,131	EC	red peppers
Methyl parathion	108	71,83	91,105	FPD	red peppers
Dacthal	48	77,83	68,74	EC	eggplants
p,p'-Methoxychlor	329	88,102	81,92	EC	eggplants
Ethion	270	101,108	95,102	FPD	eggplants
Lindane	200	81,92	80,87	EC	dried beans
Heptachlor	175	79,93	82,95	EC	dried beans
Parathion	394	90,105	97,111	FPD	dried beans

[a] CAS Registry numbers: chlorpyrifos, 5598-15-2; diazinon, 333-41-5; nitrofen, 1836-75-5; quintozene 82-68-8; vinclozolin, 63207-24-9; methyl parathion, 298-00-0; dacthal, 186-32-1, *p,p'*-methoxychlor, 72-43-5; heptachlor, 76-44-8. For ethion, lindane, and parathion, see footnote *a* in Table 1.

lyzed in 4–15 laboratories. The mean recovery was reported as 97% with a range of 70–113%. The composite CV was 13.5% with a range of 4–30%.

It is very difficult to state absolute criteria for acceptability of an analytical method. In pesticide residue analysis, recoveries of 80–110% are generally considered acceptable (10). A CV of 15% or better is suggested as a benchmark against which to evaluate results of interlaboratory studies. Precision of repeat determinations by one analyst within a laboratory should be one-half to two-thirds of the interlaboratory CV (8, 9). The automated cleanup system produced results that are generally within the criteria suggested for acceptability of pesticide residue methods.

The recovery data for various pesticides in cucumbers, red peppers, eggplants, and dried beans are presented in Table 2. The pesticides used in this study were chosen on the basis of their frequency of occurrence during routine surveillance of imported fresh fruits and vegetables between 1983 and 1984. Commodities were chosen on a similar basis. Recoveries for the manual method ranged from 71 to 117% (average 91%); for the automated system they ranged from 68 to 131% (average 95%). In addition, these data demonstrate that the automated system is essentially free of flame photometric and electron capture artifacts.

It is important to determine percent recovery of hexane in the solvent partitioning step, as this has an important effect on recovery. We determined a percent hexane recovery of 85% for the high moisture products and 70% for the dried beans. These values will vary from system to system and will also depend on the age and condition of the pump tubing in the system.

We have not yet evaluated the system for analysis of fruits. For intermediate and high sugar content products, the water-acetonitrile phase may tend to separate while the samples are waiting to be aspirated into the SPM.

Acknowledgments

The authors express their appreciation to Technicon Industrial Systems (TIS) for making available most of the equipment used in this study and to the following TIS staff members for their invaluable advice and assistance: Tony Pietrantonio, Donald Burns, Jose Fernandez, Walter Ehoodin, and Charles Weiss.

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SPICES

UV Spectrophotometric Determination of Piperine in Pepper Preparations: Collaborative Study

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Eight collaborating laboratories performed replicate analyses for piperine on 5 samples representing pepper raw spice, oleoresins, and soluble seasonings. Piperine is extracted into ethylene dichloride and measured at maximal absorbance 342-345 nm with a UV light source. Piperine content is calculated using an absorbance factor derived from piperine. Intralaboratory coefficients of variation (CV_O) ranged from 0.5 to 3.1%; interlaboratory coefficients of variation (CV_x) ranged from 3.0 to 5.8%. The method has been adopted as an official method of the American Spice Trade Association and as an official first action method by AOAC.

Pepper, deemed the world's most important spice, is produced from the small round berries of a woody perennial evergreen climbing vine, *Piper nigrum* (1). Its distinctive piquant flavor is due to piperine (2). Procedures currently used to establish piperine content include nitrogen (Kjeldahl) determination (3, 4) and colorimetric (5) methods. Problems exist with the modified nitrogen methods because adulteration with suitable organic nitrogen compounds cannot be detected (6), and in the colorimetric method, reactions with carbohydrate carriers used in soluble seasonings give piperine values in excess of actual levels present (7). The method presented here, similar to methods previously reported in the literature (8–11), provides a relatively quick and simple way to establish the spice heat constituent in pepper. The method can be applied as a practical assay for laboratories involved with many samples on a routine basis.

Collaborative Study

The method proposed by the American Spice Trade Association (ASTA) Technical Group was submitted to 8 collaborating laboratories along with 5 samples of various pepper preparations and a piperine standard. The laboratories were required to determine an absorbance factor using the piperine standard and to run each sample in duplicate.

Samples for the collaborative study were prepared as follows: black pepper raw spice (samples 1, 2) were ground in a Retschmill; black pepper oleoresins (samples 3, 4) were heated to 40°C, stirred, and split; black pepper soluble seasoning (sample 5) was prepared in a Hobart mixer as a dispersion on dextrose.

Piperine in Pepper Preparations

Spectrophotometric Method

First Action

American Spice Trade Association-AOAC Method

Principle

Piperine is extd into $C_2H_4Cl_2$ and UV absorption is measured at max. 342–345 nm. Note: Method also dets other piperine isomers that may be present as well as related compds, such as piperettine and piperylin, that also absorb at 340–345 nm.

Apparatus and Reagents

(a) *UV spectrophotometer.*—Beckman A-25, or equiv., with 1 cm sq. silica cuvets.

(b) *Volumetric flasks.*—100 mL g-s, amber (No. 161-9057, Lurex Scientific, PO Box 2420, South Vineland, NJ 08360). Amber flask is required to reduce photodegradation of piperine in solution.

(c) *Ethylene dichloride.*—Reagent grade (J. T. Baker). Make all dilns with this solv.

(d) *Piperine.*—Aldrich Chemical Co., No. P4,900-7.

Determination of Absorbance Factor

Weigh 0.1000 g piperine into 100 mL vol. flask, add ca 70 mL $C_2H_4Cl_2$, shake to dissolve, and dil. to vol. Pipet 10 mL into vol. flask and dil. to vol. Into six 100 mL vol. flasks, pipet 1, 2, 3, 4, 5, and 6 mL aliquots, resp., and dil. each to vol. Zero spectrophtr with $C_2H_4Cl_2$ and read A of each final diln at max. 342–345 nm using UV light source and $C_2H_4Cl_2$ in ref. cell. Divide each reading by mL used in final diln to convert A to concns of $1/10^6$ (μg/L). Det. av. of 6 values. Reciprocal of this av. is concn, mg/mL, of piperine required for A of 1.000, and is factor (F) used for calcns.

Procedure

(a) *Black and white pepper.*—Grind and screen sample to pass 30 mesh NBS sieve and blend uniformly. Accurately weigh (±0.0001 g) 0.5 g sample and transfer to 125 mL Erlenmeyer. Protect from light. Add ca 70 mL $C_2H_4Cl_2$. Reflux and stir 1 h, cool to room temp., and filter quant. thru paper into 100 mL vol. flask. Transfer rest of extd residue to filter, wash thoroly, and dil. to vol. Pipet 2 mL of this soln into 100 mL vol. flask and dil. to vol. Record A at max. 342–345 nm.

(b) *Pepper oleoresins.*—Accurately weight (±0.0001 g) 1.0 g well mixed sample and transfer to 100 mL vol. flask. Dil. to vol. with $C_2H_4Cl_2$. Shake well until dissolved. Pipet 10 mL into 100 mL vol. flask and dil. to vol. Pipet 1 mL of this soln into 100 mL vol. flask and dil. to vol. Record A at max. 342–345 nm.

(c) *Soluble pepper seasonings.*—Accurately weigh (± 0.0001 g) 2.0 g sample and transfer to 100 mL vol. flask. Add ca 70 mL $C_2H_4Cl_2$ and let stand 20 min, swirling occasionally. Dil. to vol. and let settle. Pipet 1 mL into 100 mL vol. flask and dil. to vol. Record A at max. 342–345 nm.

Calculations

$$\% \text{ Piperine} = [(A_s \times F \times V)/(W_s \times 10^6)] \times 100$$

where A_s = absorbance of sample; F = factor derived from piperine std; V = diln vol., mL; W_s = sample wt, g.

CAS-94-62-2 (piperine)

Results and Discussion

Results were received from 8 collaborating laboratories (Table 1). One laboratory spilled one sample of the pair of both samples 1 and 2. No laboratory reported difficulties with the procedure. Table 1 also includes the statistical analysis which was performed following methods in the *Statistical Manual of the AOAC* (12). The results of Laboratory 6

Submitted for publication June 27, 1986.
The recommendation of the Associate Referee, T. Lupina, was approved by the General Referee and the Committee on Foods II and was adopted by the Association. See the General Referee and Committee reports, *J. Assoc. Off. Anal. Chem.* (1987) **70**, March issue.

Table 1. Collaborative data for UV estimation of piperine (% by weight)

	Sample[a]				
Coll.	1	2	3	4	5
1	7.76	5.01	51.7[b]	39.1	2.05
	7.64	4.96	48.7	38.1	2.04
2	7.24	5.17	48.0	38.6	1.96
	7.30	5.20	48.1	39.0	2.00
3	7.42	4.96	48.7	39.1	2.00
	8.41[b]	4.47	49.5	39.7	2.06
4	7.40	4.62	48.2	39.5	1.96
	7.42	4.64	48.5	39.6	1.94
5	6.87	4.31	45.2	43.5	2.13
	7.08	4.53	45.1	43.9	2.02
6	7.12	4.61	48.2	38.5	1.96
	—[c]	—[c]	48.2	38.4	1.99
7	7.58	4.87	48.7	40.0	1.90
	7.42	4.80	48.9	39.0	1.85
8	7.11	4.52	49.6	40.5	2.04
	7.10	4.55	49.8	40.9	2.07
Mean, % piperine by weight	7.30	4.74	48.19	39.84	2.00
Repeatability[d]					
S_o	0.086	0.146	0.224	0.423	0.037
CV_o, %	1.2	3.1	0.5	1.1	1.8
Reproducibility[e]					
S_x	0.266	0.276	1.469	1.741	0.071
CV_x, %	3.6	5.8	3.0	4.4	3.6

[a] Each result is one of duplicate determinations. See text, *Collaborative Study*, for sample descriptions.
[b] Outlier by Cochran test. Both results on this sample excluded from statistical evaluation.
[c] Sample lost.
[d] Intralaboratory variation. Laboratory 6 not included in this evaluation.
[e] Intralaboratory evaluation.

were not included in the repeatability calculations, but were included in the reproducibility calculations. Laboratory 3, sample 1, and Laboratory 1, sample 3, were determined to be outliers.

This method may overestimate the piperine or "spice heat constituent" in pepper because other piperine isomers (isopiperine, chavicine, isochavicine) also absorb in the 340–345 nm range. These compounds are present in pepper and pepper extracts, particularly those exposed to light (13). To minimize conversion to these compounds, this method specifies amber glassware and other protection from light exposure.

Other related compounds, such as piperettine and piperylin, also absorb at 340–345 nm and also give incorrect high "piperine" values (14). These shortcomings should be kept in mind by those who require a more precise measure of piperine, but should not prevent use of this method by laboratories requiring a practical analysis method.

Recommendation

It is recommended that the UV spectrophotometric method for the determination of piperine in pepper preparations be adopted official first action.

Acknowledgments

The authors thank Susan Dingee for technical assistance in sample preparation and the following collaborators and their associates for their cooperation in the study:

R. Amaducci, Norda, Inc., East Hanover, NJ
W. Brittin, McCormick-Stange, Chicago, IL
B. Coyle, Fritzsche, Dodge & Olcott, New York, NY
A. Manheimer, J. Manheimer, Inc., Long Island City, NY
R. Meer, Meer Corp., North Bergen, NJ
D. Rone, Griffith Laboratories, Alsip, IL
R. Ruggles, OM Ingredients Co., Madison, WI
S. Sica, Commodity Laboratories, Inc., New York, NY

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PRESERVATIVES

Determination of Sulfur Dioxide in Foods by Modified Monier-Williams Distillation and Polarographic Detection

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A rapid, sensitive polarographic method is presented for determining sulfiting agents in foods and beverages. The method is based on the modified Monier-Williams distillation followed by polarographic detection by differential pulse polarography or square wave voltammetry. A clearly defined wave is obtained by both techniques, with a current maximum at a potential (E) of about −600 mV vs an Ag/AgCl reference electrode. The reaction is based on the reduction of sulfur dioxide at a dropping mercury electrode. Peak current was linear over the range 0-20 μg/mL. Quantitation is done by linear regression analysis of standard addition data or by using a standard calibration graph. Screening levels of less than 1 ppm total SO_2 were easily achieved in the foods analyzed, which had levels from less than 1 ppm (cereals) to thousands of parts per million (dried fruit). Recoveries from fortified samples ranged from 70 to 108% at fortification levels of 20, 100, and 1000 μg/g.

Sulfiting agents have been used as preservatives in food for many years. Their inhibitory spectrum extends to some yeasts, molds, and bacteria. Sulfiting agents also inhibit enzymatic and nonenzymatic activity, which causes some browning in foods. Sulfites can cause adverse reactions among sensitive individuals. To protect the consumer, the U.S. Food and Drug Administration has proposed that processed-food labels disclose the presence of sulfites whenever they are detectable in the finished product. Ten parts per million is proposed as the lowest detectable amount (1).

When added to foods, sulfites covalently bind to various functional groups of many compounds present, such as carbonyl groups, sugars, color components, phenolic compounds, and unsaturated compounds. The overall effect of this binding is a decrease in the amount of undissociated sulfurous acid, which has preservative effects.

Several laboratories are studying the behavior of sulfite addition compounds in solution (2–4). For legislative purposes, the official AOAC methods for determining total sulfur dioxide content in foods are used. These include a direct colorimetric method for determination of sulfur dioxide in beer (5) and dried fruit (6); and the indirect Monier-Williams distillation method (7) for a variety of other foods. The Monier-Williams method lacks sensitivity and specificity, and is also subject to interferences from volatile components in foods such as cabbage and onions. As a result, a great number of modified methods have been published for determining free, bound, or total sulfur dioxide in food and beverage products. In a critical review published in 1977 (8), Carswell summarized the abundant analytical sulfur dioxide methodology involving direct and indirect methods with various detecting techniques. Other published methods determine sulfites as sulfur dioxide by gas chromatographic headspace detection (2, 9), direct polarographic detection in nonaqueous media (10), or modified Monier-Williams distillation with colorimetric detection (11–13). Ion chromatgraphy has also been applied to analyze complex food materials (14).

The purpose of this work was to develop a reasonably fast screening method applicable to determining sulfur dioxide in foods at part per million screening levels. Combining the classical distillation method for isolation of SO_2 from foods and the modern polarographic detection technique gives the analyst a very effective and rapid screening method with detection of less than part per million levels.

Received December 2, 1985. Accepted April 2, 1986.

METHOD

Reagents

(a) *Phosphoric acid.*—85%. Analytical reagent grade.

(b) *Diluting solution.*—5% (v/v) methanol used after deaeration.

(c) *Supporting electrolyte and trapping buffer.*—1M ammonium acetate–acetic acid. Dissolve 77.1 g analytical reagent grade ammonium acetate in ca 400 mL water, and add 57 mL glacial acetic acid. Mix and dilute to 1 L with diluting solution (b).

(d) *Sulfite stock standard solution.*—1000 μg SO_2/mL. Determine SO_2 content of analytical reagent grade sodium metabisulfite ($Na_2S_2O_5$) or sodium bisulfite ($NaHSO_3$) by AOAC method **10.086** (5). Weigh and dissolve 1.484 g $Na_2S_2O_5$ in 1 L diluting solution.

(e) *Working standard.*—100 μg SO_2/mL. Dilute 10.0 mL stock standard solution to 100.0 mL with diluting solution. Prepare fresh daily.

(f) *Silicone defoamer.*—Dow Corning Antifoam A spray or oil.

Note: None of the reagents used are known to pose any significant safety hazard.

Apparatus

(a) *Polarographic system.*—EG&G Princeton Applied Research (PO Box 2565, Princeton, NJ 08540) Model 384B system consisting of Model 384B polarographic analyzer, Model 303 static mercury dropping electrode equipped with silver/silver chloride reference electrode, and Houston Instrument DMP-40 plotter (8500 Cameron Rd, Austin, TX 78753).

(b) *Distillation apparatus.*—As shown in **20.124**, Figures **20:05** and **20:06** (7), or equivalent.

(c) *Compressed nitrogen*—AGA Gas Inc., Specialty Gas, 6421 Monclova, Maumee, OH 43537, or equivalent, equipped with flow regulator set at 0.5 L/min.

(d) *Electric heating mantle and mantle top.*—(Glas-Col Apparatus Co., PO Box 2128, Terre Haute, IN 47802.) Regulated by rheostat (Powerstat Variable Autotransformer, The Superior Electric Co., 383 Middle St, Bristol, CT 06010).

(e) *Circulating chilled water bath.*—(Haake FK, Saddle Brook, NJ 07662.)

(f) *Homogenizers.*—Waring blender, Polytron (Brinkmann Instruments), Hobart food cutter (Hobart Corp., Troy, OH 45374).

Sample Preparation

Beverages.—Mix cold beverage in closed container before removing portion for analysis.

Table 1. Instrument parameters for polarographic Model 384B system

Mode	SWV	DPP
Initial E, V	−0.400	−0.400
Final E, V	−0.800	−0.800
Pulse height, V	0.020	0.020
Purge time (initial), s	240	240
Purge time (between detns), s	240	240
Equilibration time, s	3	N/A
Frequency, Hz	20	N/A
Scan increment, mV	2	2
Scan rate, mV/s	40	N/A
Cycles	1	N/A
Replications	1	1
SMDE (drop size)	small	small
Drop time, s	N/A	0.5

Soup concentrates.—Homogenize by Polytron or blender.

Potato salad and cole slaw.—Homogenize in blender.

Raw vegetables.—Cut into cubes of ca ½ in., and mix.

Canned vegetables.—Cut into cubes of ca ½ in., and mix.

Raw or cooked shrimp.—Thaw, drain liquid, mix, and pass representative portion through meat grinder.

Dried fruit and nut mixes.—Mix and pass through food grinder.

Procedure

Assemble apparatus as described in **20.124** and shown in Figures **20:05** and **20:06** (7) in fume hood using alternative SO_2 trapping apparatus introduced by Thrasher (15). Prepare trapping assembly (A) and (B) with 10.0 mL and 5.0 mL, respectively, of trapping buffer. Circulate cooled water in condenser maintained at ca 5°C. Optimize polarographic system and determine its reproducibility by square wave voltammetry (SWV) or differential pulse polarography (DPP).

Weigh and transfer representative portion of sample composite (usually 25.0 or 50.0 g) into semimicro blending cup. Add 100.0 g diluting solution and blend until homogeneous. Transfer 10.0 g blended composite to 3-neck 250 mL distilling flask. Add diluting solution to bring total weight to 20 g. Add antifoam and connect apparatus. Purge system with nitrogen for 2 min then introduce 5 mL phosphoric acid through dropping funnel and attach heating mantle and cover preheated to 100°C. Distill 10 min after steady refluxing state is reached, then remove Kuderna-Danish trapping assembly (A), rinse scrubber tube, and replace with additional assembly (B). Distill 5 min while performing polarographic determination on trapping solution (A). Then, test solution (B) for residual SO_2.

Table 2. Recovery by the proposed method of total SO_2 from fortified foods, using 2 different trapping solutions

			0.1N NaOH trapping soln		1M ammonium acetate–acetic acid trapping soln	
Food	Initially found, ppm[a]	$Na_2S_2O_5$ added, ppm	Total rec., ppm[a]	% Rec.	Total rec., ppm[a]	% Rec.
Frozen shrimp	79.2	100.0	155.9	87.0	166.7	93.0
Yellow canned hominy	149.4	100.0	240.6	96.5	242.2	97.1
Soy powder	105.2	100.0	197.5	96.2	196.1	95.6
Dark raisins	0.00	100.0	98.4	98.4	98.2	98.2
Candied pineapple	167.0	100.0	267.5	101.2	266.5	99.8

[a] Average of 2 determinations.

Table 3. Recovery of total SO_2 by the proposed method (single determinations) from fortified food materials

Food	Initially found, ppm	Added, ppm	Total rec., ppm	Total rec., %
Condensed clam chowder soup	0.30	20.0	19.97	98.4
Raw potatoes (packaged)	5.01	20.0	24.9	99.4
Potato salad	7.0	20.0	26.2	96.0
Banana chips	0.9	20.0	18.1	86.0
Fruit and nut mix	10.5	20.0	25.7	76.0
Raisin-date granola	0.1	20.0	14.1	70.0
Frozen raw shrimp	10.1	20.0	26.4	81.5
Onion rings	0.00	100.0	81.6	81.6
Frozen blueberries	0.00	100.0	97.4	97.4
Fruit and nut mix	167.0	100.0	249.6	82.6
Cherry drink	42.1	100.0	150.1	108.0
White grape juice	50.0	100.0	152.0	102.0
White canned hominy	117.8	100.0	211.0	93.2
Soy powder	101.6	100.0	180.4	78.8
Black raisins	0.0	1000.0	1018.0	101.8
Frozen raw shrimp	10.1	1000.0	980.0	98.0
Papaya spears	168.0	1000.0	1172.0	100.4

Polarographic Determination

Determine blank by DPP or SWV technique. Transfer 10.0 mL solution containing buffer and sample to polarographic cell. Purge 4 min and obtain polarogram (voltammogram). If sample solution is very concentrated, add portion via micropipet to measured buffer in cell or make dilution with deaerated trapping buffer. Use linear regression analysis of the standard addition data for quantitation. Treat concentrations of standards and their corresponding diffusion currents as the abscissa-ordinate pairs. *X*-Axis intercept provides concentration of unknown. Instrumental conditions are given in Table 1. If preferred, prepare calibration graph in 0–20 μg SO_2/mL concentration range and determine sample concentrations graphically.

Recovery Experiments

Recovery experiments were performed on 10.0 g sample composites of various food materials by fortifying them with appropriate aliquots of stock standard solution. Spiking solution soaked into food materials about 15 min before analysis. Recoveries were measured at 3 levels of fortification: 20, 100, and 1000 μg SO_2/g sample.

Results and Discussion

Earlier studies involving square wave and differential pulse polarographic determinations of SO_2 in foods were done on simple food materials, such as fruit-flavored drinks, fruit juices, applesauce, or pickling solutions (16). Determinations involved extraction of sulfiting agents from food matrix by using various buffer systems and direct determination of SO_2

Table 4. Determination of total SO_2 in dried fruit by the proposed method and by the AOAC colorimetric method (6)

Food	Proposed method, ppm	AOAC method, ppm
Dark raisins	0.00	inconclusive
Dried peaches	2580.0	2500.0
Papaya spears	480.0	468.5
Light seedless raisins	1467.0	1502.0
Pineapple chunks	131.3	122.0
Dried fruit mix	398.0	402.5
Pitted dates	0.00	0.00
Dried apples	106.0	100.0

Table 5. Repeatability of total SO_2 determinations on white canned hominy[a]

Detn	Total SO_2 found, ppm
1	110.3
2	109.7
3	110.1
4	109.8
5	110.0
Mean	110.0
SD	0.24
% RSD	0.22

[a] Sample wt, 10.0 g.

by SWV or DPP analysis. This direct preliminary screening is still used for juices, fruit drinks, and pickling solutions. For example, analysis of pickling solution from canned cauliflower buds gave 880 μg SO_2/mL, while analysis of cauliflower buds alone by the proposed method gave 800 μg/g. Sample weight and the volume of solution in the polarographic cell were adjusted accordingly to avoid "over-loading" the signal.

The proposed method can be considered a 4-phase operation: (*1*) sample preparation, (*2*) release of SO_2 from acidified solution and carry-over into the trapping vessel, (*3*) absorption of SO_2 into the trapping solution, and (*4*) quantitation of SO_2 by DPP or SWV analysis.

Sample size and different ways of "stabilizing" the sample against air oxidation were investigated during method development. Sample size was usually 10 g. Liquids were mixed, and solids were homogenized with deaerated water containing methanol. The compositing step was carried out as a nonstop operation and distillation was started immediately. Stabilizing and extracting SO_2 from foods by using buffer solutions has been reported by Hamano et al. (17) for determination of free and combined sulfites in foods. Alkaline glycerol buffer was used by Lloyd and Cowle to prevent SO_2 oxidation while performing direct iodometric titration (18). Tartrate, citrate, or phosphate pH 4.1 buffers have been used as extractants or stabilizers. At this pH, 99.5% sulfite is present as bisulfite, and losses due to volatilization are reduced. Holak and Patel (19) use pH 4.4 glycerol–acetate buffer for homogenizing the sample prior to acidification and desorption. Glycerol is used for stabilizing stock standard solutions. The proposed method uses methanol as a stabilizing reagent against air oxidation of standards and food materials. Stock standard solutions stabilized with 5% methanol were stable in closed containers for several days. Methanol is one of the substances that inhibit the bisulfite oxidation process by breaking the reaction chain mechanism (20). No decomposition of SO_2 due to light was observed.

Release of SO_2 from sample composites is carried out using phosphoric acid. Phosphoric acid was chosen because it is less volatile than HCl, which reduces its carry-over into an absorption buffer.

Nitrogen carrier gas flow is another factor that influences recoveries of SO_2 from foods. The flow must be rapid enough to carry SO_2 into the trapping solution but not so fast as to reduce the amount of contact between the gases and the absorbing medium. Nitrogen flow in the proposed method should be maintained at about 0.5 L/min.

Other analysts have used various SO_2 trapping solutions in distillation and desorption procedures (2, 21). The proposed method uses 1M ammonium acetate–acetic acid buffer, which has been found applicable for trapping SO_2 (personal communication, L. E. Habeger, FDA, Minneapolis, MN). This is the same reagent as the polarographic electrolyte, so it expedites the determinative step because no pH adjustments are needed. Spiked sample recoveries obtained with 1M ammonium–acetic acid buffer are close to those obtained using 0.1N NaOH as trapping medium as shown in Table 2.

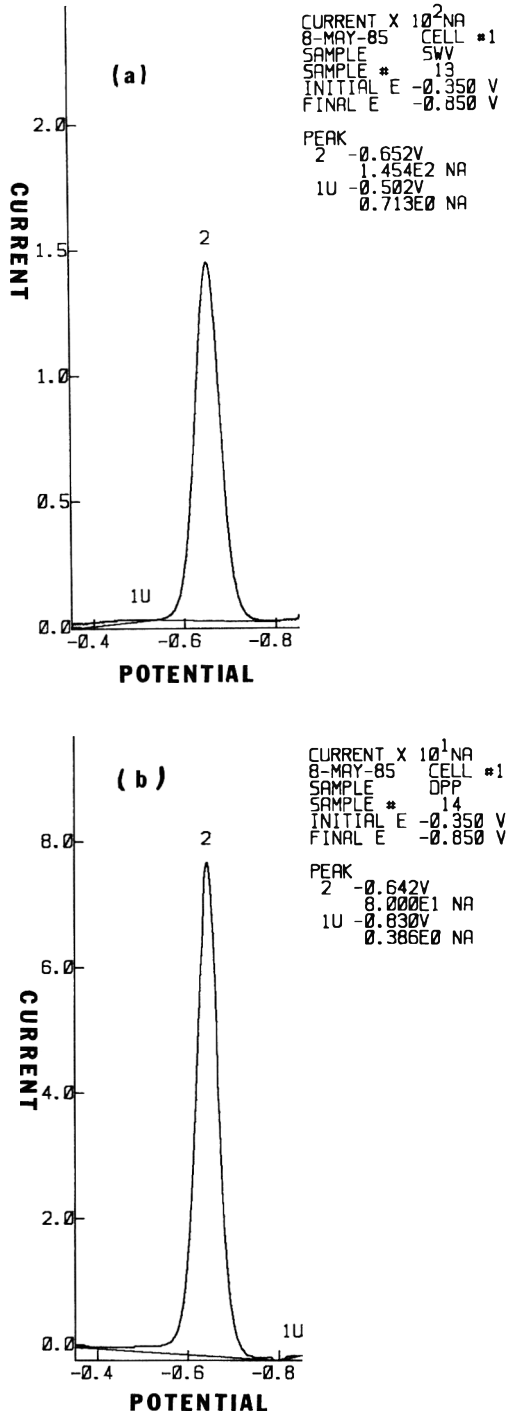

Figure 1. Determination of SO_2 in coconut candy by distillation polarographic method (0.3 g sample in polarographic cell, 0.35 ppm SO_2 found). (a) SWV procedure; (b) DPP procedure.

Table 3 summarizes results of all recovery experiments performed in this study. The determinative step was SWV analysis.

Results for fortified samples (Table 3) are based on single determinations on separate sample portions during routine sample analysis. A limited study was conducted to compare the results obtained by the proposed method with those ob-

tained by the AOAC method for dried fruit (6). The data are given in Table 4. Repeatability data are presented in Table 5 on a composite of white hominy. Reproducibility on 5 determinations was good (% RSD = 0.22). Homogeneity of sample composite is an important factor in method variability. For some products such as dried fruits or fruit and nut mixes, obtaining homogeneous composites presents more difficulty and results in larger RSD values.

Polarographic analysis by 2 techniques, square wave voltammetry and differential pulse polarography, are shown in Figures 1a and 1b, respectively. Preliminary work was done using a PAR 174 (EG&G Princeton Applied Research) polarograph with the DPP technique.

The availability of the PAR 384B microprocessor-based system makes the square wave technique accessible to a routine analytical laboratory. The attractive features of square wave voltammetry are (*1*) better sensitivity than for differential pulse polarography, (*2*) speed (analysis time measured in the order of seconds), and (*3*) use of the single drop technique (no stirring problems as associated with the DPP procedure). The polarographic system was usually optimized by measuring 2 buffer blanks and 6 replicate standard determinations. If the RSD for 6 replicate standards was less than 2%, polarographic system precision was considered satisfactory. Using 1-cycle determinations by the SWV technique, the correlation coefficient during the quantitation step was 0.999 or better. During sample determination, precautions were taken to keep sample solutions tightly stoppered until polarographic work was completed.

The proposed method determines sulfites in various foods as total SO_2. The method is moderately fast, very sensitive, and specific for SO_2, and appears to lack many common interferences encountered in the Monier-Williams method. Due to high acidity and temperature, hydrolysis of bound forms of sulfur dioxide occurs. It was not within the scope of this work to include the procedure for determining free or unbound SO_2. Such methods are of special interest to analytical chemists employed in beer and wine manufacturing industries (22). Modified Monier-Williams distillation apparatus could be used under different experimental conditions for the determination of free sulfite.

Acknowledgment

The author thanks James Howell, FDA Detroit District Science Advisor, for helpful suggestions while reviewing the manuscript, and Sophie Ramoskas for typing the manuscript.

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ELEMENTAL ANALYSIS

Measurement of Trace Levels of Total Aluminum in Foods by Atomic Absorption Spectrophotometry

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A graphite furnace atomic absorption spectrophotometry method was used to determine the total aluminum content of various foods found in an average American diet. The food products were ashed in platinum dishes, and the inorganic residue was fused using a sodium carbonate-sodium borate mixture. The fusion step allowed detection of all the various forms of aluminum found in food products. A L'vov platform was used in the graphite furnace to increase the sensitivity of the assay. Care was taken throughout the analysis to avoid various sources of aluminum contamination such as glass and porcelain dishes. All reagents were ultra-pure grade and were continuously monitored for aluminum content. Sodium borate used in the fusion flux mixture had previously been extracted with 8-hydroxyquinoline in chloroform to remove any aluminum present. Both raw and cooked foods were analyzed for aluminum with this method. Average recoveries of aluminum from food products ranged from 84 to 112%. The overall coefficient of variation of this method on the food products tested was 10%.

Aluminum and the role it plays in the human body have been topics of interest over the past several years. Studies have been conducted to determine the various pharmacological effects of dosed aluminum (1–4), to correlate the elevated aluminum levels in the brain with the onset of Alzheimer's disease (5), and to monitor the aluminum content of foods and spices (6, 7). Unintentional aluminum contamination from foil, cookware, and containers has also been investigated (8–10). With the increased activity in monitoring aluminum intake and its subsequent bioavailability in humans, a sensitive method to measure aluminum accurately is needed.

The purpose of this study was to develop a sensitive procedure that could accurately measure the total aluminum content in a cross section of foods in the American diet.

The method chosen for quantitation was graphite furnace atomic absorption spectrophotometry (11, 12). This method was selected for sensitivity, speed of analysis, and flexibility in sampling techniques. Classic flame atomic absorption procedures are capable of determining 2–5 parts per million (ppm) aluminum in food. In this study we examined aluminum levels below 1 ppm.

METHOD

Apparatus

(a) *Atomic absorption spectrophotometer.*—Perkin-Elmer Model 4000.

(b) *Graphite furnace.*—Perkin-Elmer Model HGA-400.

(c) *Autosampler for graphite furnace.*—Perkin-Elmer Model AS-40.

(d) *Graphite tubes.*—Pyrolytically coated, Perkin-Elmer.

(e) *L'vov® platforms.*

(f) *Aluminum hollow cathode lamp.*—Perkin-Elmer.

(g) *Deuterium arc lamp.*—Background correction, Perkin-Elmer.

Received December 19, 1985. Accepted May 9, 1986.

Reagents

(a) *Aluminum reference standard.*—1000 ppm, Fisher Certified.

(b) *Aluminum working standards.*—0.01, 0.03, 0.05, 0.10, 0.30, 0.50, 1.00, 3.00, and 5.00 ppm made with 5% (v/v) HNO_3 and 2% (w/v) sodium carbonate–sodium borate mixture by dilution from stock standards.

(c) *Sodium carbonate–sodium borate mixture (3 + 1).*—Weigh 75 g sodium carbonate and 25 g sodium borate and transfer to plastic bottle. Mix thoroughly by shaking and inverting for several minutes. Extract sodium borate with 8-hydroxyquinoline in chloroform before mixing with sodium carbonate.

(d) *Nitric acid, concentrated.*—Ultrex® ultra-pure grade.

Sample Preparation

Most foods were analyzed in the raw state. When cooking was necessary (e.g., rice, pudding), foods were prepared in stainless steel cookware according to directions from a commercial cookbook (13). Cooking was done in a separate room isolated from the analytical laboratory. Double-deionized water was used to prepare food when necessary. Aluminum

Table 1. Aluminum content of spices and herbs determined by emission spectroscopy[a]

Sample	Av. Al level, ppm
Allspice	80
Basil	324
Bay	474
Cardamom	83
Celery seed	417
Cinnamon	81
Clove	119
Coriander	32
Cumin	432
Dill seed	100
Dill weed	89
Fennel	67
Garlic	<10
Ginger	125
Mace	111
Marjoram	870
Mustard	<10
Nutmeg	<10
Onion	<10
Oregano	645
Paprika	303
Parsley	97
Pepper, black	138
Pepper, red	53
Pepper, white	40
Poppy seed	33
Rosemary	372
Sage	396
Savory	525
Sesame seed	<10
Tarragon leaves	316
Thyme	1000
Turmeric	570

[a] Source: Ref. 6.

Table 2. Accuracy data in determination of baseline aluminum levels and recoveries on representative foods

Matrix	Base level, ppm		Recovery, %[a]	
	Mean ± SD[b,c]	Range	Mean ± SD[b]	Range
Chocolate	11.5 ± 0.7	10.6–12.4	101 ± 6	90.8–108
Rice[d]	1.5 ± 0.2	1.4–1.8	102 ± 9	93.6–118
Pudding[e]	4.0 ± 0.3	3.8–4.5	100 ± 13	90.0–118
Grits	0.62 ± 0.18	0.43–0.84	100 ± 8	92.0–109
Smoked ham	0.85 ± 0.55	0.39–1.6	104 ± 16	79.0–124
Applesauce	0.13 ± 0.03	<0.10–0.16	107 ± 4	104–114
Fish, cod	0.35 ± 0.06	<0.30–0.44	—	—
Fish, flounder	0.55 ± 0.20[f]	0.32–0.84[f]	100 ± 9	90.0–113
Chicken	0.47 ± 0.15	<0.30–0.64	91 ± 6	83.0–97.5
Eggs	0.10 ± 0.00	<0.10	108 ± 4	103–112
Cabbage	0.13 ± 0.04	<0.10–0.18	102 ± 5	98.0–112
Peas	1.9 ± 0.1	1.8–2.0	112 ± 12	97.9–133
Green beans	3.8 ± 0.4	3.2–4.3	95 ± 12	82.5–115
Cauliflower	0.19 ± 0.09	<0.10–0.31	108 ± 4	100–112
Tomatoes	0.12 ± 0.04	<0.10–0.18	83 ± 8	77.2–92.0 (N = 3)
Potatoes	0.16 ± 0.05	<0.10–0.23	94 ± 7	87.5–101 (N = 3)
Beef, pot roast	0.19 ± 0.08	<0.10–0.31	—	—
Beef, eye of round	0.39 ± 0.14	<0.30–0.59	—	—
Beef, chuck roast	0.30 ± 0.00	<0.30	—	—
Beef, ground, raw	0.14 ± 0.05	<0.10–0.22	93 ± 13	77.2–115 (N = 8)
Beef, ground, cooked	—	—	101 ± 12	84.2–118 (N = 9)
Tomato sauce	0.10 ± 0.01	<0.10–0.13	—	—
Spaghetti	1.7 ± 0.3	1.4–2.0	111 ± 19	77.0–128
Turkey	0.32 ± 0.04	<0.30–0.41	—	—
Overall average recovery, %			101 ± 7	77.2–133

[a] Recoveries were performed at one-half, one, and 2 times the baseline level of aluminum found in each food product.
[b] N = 6.
[c] Values of <0.10 and <0.30 ppm were rounded to 0.10 and 0.30, respectively, for calculations.
[d] Uncooked rice was soaked in water.
[e] Pudding was hydrated and cooked in a stainless steel pan.
[f] N = 12.

content of the water was measured before each use; levels were all less than 0.01 ppm.

All vegetable, fruit, and cereal products were homogenized in a stainless steel blender; meat and fish were passed through a stainless steel grinder. Chocolate bars and dry chocolate pudding mix were homogenized by blending with dry ice. All foods were processed until a uniform product was obtained. After homogenization, samples were placed in double polyethylene bags and aliquots were removed for aluminum analysis. All remaining food samples were stored frozen in double polyethylene bags. Storage in polyethylene bags and the short time lapse between preparation and analysis ensured sample homogeneity.

Aluminum Analysis

Weigh 10–50 g of each food sample (depending on type) into platinum dish and char on hot plate. Ash samples at 500–600°C for 6–8 h. After ashing, fuse samples at 1000°C for 10 min with 1 g sodium carbonate–sodium borate mixture (3 + 1).

Boil fused samples in 5 mL concentrated HNO_3 and transfer to 50 mL volumetric flask. Final sample matrix is 2% fusion flux and 10% HNO_3.

Prepare aluminum standards with same fusion flux and acid composition as samples. Using same matrix for samples and standards compensates for suppression effect that occurs on aluminum signal in graphite furnace atomic absorption spectrophotometry.

Prepare 2 standard curves. Use low standard curve for quantitating sample solution concentrations from 0.01 to 0.5 ppm; use high standard curve to quantitate sample solution concentrations ranging from 0.1 to 5.0 ppm.

Inject solutions into graphite furnace under the following instrument conditions. Determine amount of aluminum present by comparing absorbance produced by each sample with that produced by standards of known concentrations.

Instrument Conditions

Graphite furnace protocol.—Wavelength, 309.3 nm; slit width, 0.7 nm, low; lamp current, 25 mA; graphite tube, pyrolytically coated with L'vov platform; integration time, 3.0 s; signal, absorbance, peak height; mode, background correction, rec abs-1; injection volume, 5 μL high curve 15 μL low curve; replicate injections, 3; gas interrupt—miniflow, 200 mL/min low curve, continuous flow high curve.

Temperature program.—As follows:

Step	*Function*	*Temperature, °C*	*Ramp time*	*Hold time*
1	Drying	Ambient to 150	15	15
2	Ashing	1000	20	15
3	Atomization	2600	0	3
4	Burn out	2600	0	3
5	Cool down	20	2	8

Results and Discussion

Early work to determine aluminum in foods was conducted by ashing the sample followed by flame or graphite furnace atomic absorption spectrophotometry. Analysis of food samples for aluminum content using flame atomic absorption provided a detection limit of 2–5 ppm. Almost all food samples investigated in this study contained aluminum at levels below the limit of detection of this technique. To quantitate these trace levels of aluminum in food, an improved graphite furnace atomic absorption procedure was developed.

Parallel investigations showed that various forms of alu-

Table 3. Precision data for aluminum levels in beef

Replicate	Al level, ppm	
	Raw	Cooked in Al
1	0.172	5.57
2	0.170	6.48
3	0.161	7.05
4	0.179	7.15
5	0.182	5.90
6	0.190	6.26
7	0.185	7.55
8	0.177	7.45
9	0.167	—
10	0.174	—
Mean	0.176	6.68
SD	0.0087	0.733
CV, %	4.96	11.0

minum typically found in foods, such as aluminum oxide and aluminum silicate, could not be recovered unless samples were fused before quantitation. Sodium metaborate, sodium borate, sodium hydroxide, and sodium peroxide were investigated as possible fusion mixtures. A combination of sodium carbonate and sodium borate proved most successful in fusing all forms of aluminum in the inorganic residue. Sodium borate, which was contaminated with aluminum, was extracted with a series of solvents as outlined in Minczewski et al. (14), and 8-hydroxyquinoline in chloroform was the most effective for extracting aluminum from the sodium borate. Levels of aluminum found in the fusion mixture and the analytical reagents are as follows: ultra-pure nitric acid, 0.002; deionized water, <0.001; sodium carbonate, <0.1; sodium borate (before extraction), 6–7; sodium borate (after extraction), <0.1 ppm aluminum. Recovery studies showed that, without fusion, no recovery of aluminum oxide or aluminum silicate was possible. With fusion, 98% of aluminum oxide and 100% of aluminum silicate were recovered.

The large amount of sodium from the fusion mixture that was added to each food sample strongly suppressed the absorbance of aluminum (20% suppression of signal) in the graphite furnace. To compensate for this suppression, the standard solutions were prepared using the same ratios of the fusion mixture contained in the samples. Linear responses were still produced throughout the standard curve. Use of a L'vov platform in the graphite tube increased both the sensitivity of the assay and the longevity of the graphite tube.

During this study, more than 3000 samples were analyzed, including meats, eggs, fruits, vegetables, fish, grains, and various high carbohydrate products. With a number of these samples, especially the vegetable and meat products, care was taken to ensure that a homogeneous aliquot was obtained. This was critical with the vegetable products because the seeds in vegetables contain high levels of aluminum (6). Spices, when used, were added with analytical precision because many spices also contain high levels of aluminum (Table 1). Baseline levels of aluminum in a variety of foods are presented in Tables 2 and 3.

Recovery studies were conducted on each of 18 different food types (Table 2). Recoveries were performed at one-half, one, and 2 times the baseline aluminum found in each food product. These recoveries ranged from 77.2 to 133% of the expected level. The average recovery for all products tested was 101%. The coefficient of variation between replicates averaged 10%.

The method presented in this paper provides a fast, accurate, and precise procedure for determining total aluminum in various food products.

Acknowledgments

The authors thank Mary Jane Ripp and Leemen Loy Shick for their analytical work during this study.

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MYCOTOXINS

Liquid Chromatographic Determination of Cyclopiazonic Acid in Poultry Meat

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A liquid chromatographic procedure has been developed for the determination of cyclopiazonic acid (CPA) in poultry meat. CPA is extracted from ground meat with chloroform–methanol (80 + 20), partitioned into 0.1N sodium hydroxide, acidified, and extracted into dichloromethane. An interfering component of meat is removed by transferring the dichloromethane extract to a minicolumn containing silica gel and washing the column with petroleum ether and chloroform. CPA is eluted with methanol–acetic acid (99 + 1), and subjected to ligand-exchange liquid chromatography. Recovery of CPA from 40 separate samples of meat spiked with CPA at levels from 0.016 to 15.6 ppm was 70.4 ± 14.1%. Analysis of meat from a chicken orally dosed with 10 mg CPA/kg body weight revealed that 14.5% of the dose was in muscle 48 h after administration.

Cyclopiazonic acid (CPA) is a toxic indole tetramic acid produced by a number of species of *Penicillium* and *Aspergillus* (1–3). The toxin has been isolated from cheese (4, 5), and from meats, including ham, sausage, and frankfurters (6). The more recent findings of CPA occurrence in peanuts (7), corn (8), and millet (9) as the results of *A. flavus* contamination indicates that the extent of potential exposure of both humans and domestic animals is greater than previously believed. However, no surveys have determined the degree of prevalence of CPA in food or feed, and few analytical methods are available for detecting the compound at this time.

The toxicity of CPA has been demonstrated in a variety of animal species, including rats (8, 10, 11), chickens (12), and pigs (13). A variety of symptoms including weight loss, diarrhea, depression, opisthotonus, convulsions, and death were observed. To further investigate the biological properties of CPA, we recently studied the uptake, distribution, and excretion of biosynthetically produced [^{14}C]CPA in rats (14). One of the major findings of this study was the high degree of distribution of CPA in skeletal muscle. After either oral or parenteral administration, as much as 50% of the radioactive dose was detected in muscle, and significant quantities (up to 10% of the dose) persisted in the muscle for as long as 72 h. If CPA distributes in a similar manner in domestic animals fed rations containing the toxin, then it is likely that humans may be exposed to the toxin through consumption of meat. It is therefore important to be able to assay meat samples for the presence of CPA when contamination is suspected.

We have developed a method for the determination of CPA in poultry meat. The method is based on ligand-exchange liquid chromatography (LC) (15), and is a modification of methods described for determining CPA in peanuts (16) and tenuazonic acid in tomato paste (17). A minicolumn cleanup step has been developed which removes interfering components of muscle at relatively little expense to recovery.

Received February 3, 1986. Accepted April 5, 1986.

METHOD

Apparatus

(a) *High-speed blender.*—Waring, explosion-proof, with 1 qt glass container and equipped with variable transformer.

(b) *Centrifuge.*—IEC Model K, Size 2, equipped with Model 392 carriers for 125 mL separatory funnels.

(c) *Separatory funnels.*—Kimble No. 45210F-125.

(d) *Rotary evaporator.*—Büchi flash evaporator with thermostatically controlled water bath.

(e) *Disposable pipets.*—Pasteur, 14.5 × 0.6 cm od.

(f) *Mixer.*—Vortex-type, or similar.

(g) *pH Meter.*—Corning Model 125, or equivalent.

(h) *Minicolumns.*—Plug Pasteur pipet with glass wool. Add Mallinckrodt SilicAr CC-7 to give 4 cm bed. Wash with 5–10 mL each of methanol, chloroform, and then petroleum ether. Force solvent through pipet with latex bulb.

(i) *0.45 μm Nylon filter.*—MSI Magna 66, Fisher No. NO4SPO4700.

(j) *Liquid chromatograph.*—Beckman Model 100A pump; Altex Model 210A sample injection valve; Hitachi Model 154 variable wavelength detector set at 284 nm; Hewlett-Packard Model 3390A integrator, or equivalent.

(k) *LC column.*—Beckman Ultrasphere ODS, 4.6 mm × 25 cm, or equivalent.

Reagents

Use reagent grade chemicals.

(a) *Solvents.*—Purchase as distilled in glass; use distilled, deionized water throughout.

(b) *Standard solutions.*—Produce and purify cyclopiazonic acid as previously described (12). Prepare standard solution of CPA in methanol (0.1 mg/mL), and confirm concentration by UV spectroscopy (molar absorptivity in methanol at 282 nm = 20 400).

(c) *LC mobile phase.*—Prepare aqueous stock solution of 10% ammonium acetate, 0.25% 4-dodecyldiethylenetriamine (Eastman Kodak), and 0.01M zinc acetate. Adjust to pH 7.3. To 100 mL stock solution, add 200 mL water, 300 mL isopropyl alcohol, and 400 mL acetonitrile. Mix, and let solution equilibrate to room temperature. Adjust volume to 1 L with water, and filter through 0.45 μm MSI Magna 66 Nylon filter.

Sample Extraction

Grind chicken breast and thigh muscle through meat grinder. Blend 50 g ground meat with 200 mL chloroform–methanol (80 + 20) plus 0.5 mL 6N HCl for 3 min at high speed. Start blender slowly with variable transformer to avoid splashing. Filter extract by gravity through fluted paper (S&S 588) into 250 mL separatory funnel. Let layers separate, and transfer 50 mL of lower layer to 125 mL centrifugal sepa-

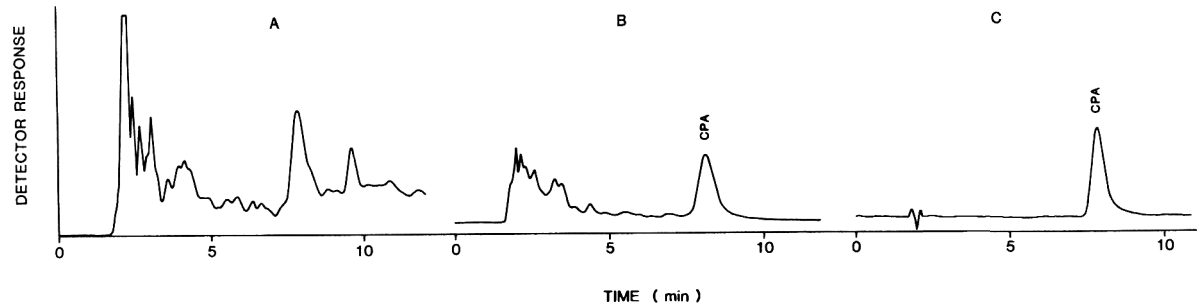

Figure 1. Liquid chromatogram of sample extracts from meat spiked with 2.0 ppm CPA: A, without minicolumn cleanup; and B, with minicolumn cleanup. C, 250 ng CPA standard.

ratory funnel. Add 50 mL 0.1N NaOH, shake solution carefully, and centrifuge at 290 × *g* for 20 min. Wash top, aqueous layer with 50 mL chloroform, and then acidify aqueous layer with 5 mL 6N HCl (check acidity with pH paper). Extract with 50 mL dichloromethane (DCM) and flash-evaporate DCM layer to near dryness.

Minicolumn Cleanup

Quantitatively transfer residue to prepared minicolumn with DCM. Wash column first with 5–10 mL petroleum ether, then with 5–10 mL chloroform. Elute CPA-containing fraction with ca 10 mL methanol–acetic acid (99 + 1), collecting eluate in 50 mL conical flask. Dry eluate by flash evaporation, then with nitrogen until odor of acetic acid is no longer detected. Dissolve residue in 0.5 mL methanol by vortex-mixing at least 1 min. Transfer solution to 5 mL disposable syringe fitted with 0.2 μm filter (Gelman Acrodisc CR, No. 4225). Collect filtrate in 1.5 mL polyethylene vial.

Liquid Chromatography

Equilibrate column with mobile phase for 5–6 h before use, recycling effluent. Use flow rate of 1.3 mL/min, with detector set for 284 nm. Inject known quantities of CPA standard solution each day to determine retention time and corresponding peak areas. Plot peak areas vs amount of CPA injected. Inject 10–50 μL sample, and adjust recorder sensitivity as appropriate for individual samples. Calculate amount of CPA per kg meat by formula:

$$\text{mg CPA/kg meat} = \frac{C \times S \times V}{Z \times W \times Y} \times 1000$$

where C = CPA area in sample aliquots; S = mg CPA standard injected; V = volume of final sample extract; Z = CPA area of standard; W = g sample represented by final extract; and Y = volume of sample injected.

Recovery

Spike chicken meat samples in blender jar with appropriate amounts of CPA standard solution prior to addition of extraction solvent. Process samples through solvent partitioning and minicolumn cleanup procedures. Determine percent recovery by comparison of LC peak areas with standard curve.

Table 1. Recovery of cyclopiazonic acid added to chicken meat

Added, mg/kg	Rec., %[a]
0.016	88.2 ± 14.1
0.156	89.3 ± 8.4
1.56	67.2 ± 8.5
7.80	81.7 ± 6.7
15.6	75.3 ± 8.1

[a] Results are means ± standard deviation of 3 separate analyses at each concentration.

Results and Discussion

The solvent partitioning cleanup procedures used both for CPA in peanuts (16) and for tenuazonic acid in tomato paste (17) included extraction of organic phases with 5% sodium bicarbonate. We found that partitioning with 0.1N sodium hydroxide gave better recoveries than partitioning with 5% $NaHCO_3$. When we added known amounts of CPA to the extraction solvent, 66% of the CPA was recovered if 5% $NaHCO_3$ was used, and 94% was recovered if 0.1N NaOH was substituted. The greater tendency for emulsions to form when 0.1N NaOH was used was overcome by using centrifugal separatory funnels.

Analyses of spiked chicken meat samples by the method described for peanuts (16), i.e., without minicolumn cleanup, yielded LC chromatograms similar to that in Figure 1A. Standard CPA eluted from the column with a retention time of 6.8–7.2 min (Figure 1C), and the presence of an interfering component in meat with a retention time slightly longer than that of CPA necessitated the development of an additional cleanup procedure. Figure 1B shows the chromatogram obtained after the minicolumn cleanup procedure described in *Method* was used. When a standard solution of CPA was applied to the minicolumn and the washing and eluting steps were carried out, 88% of the CPA was recovered from the column. Addition of acetic acid to the methanol used for elution of the minicolumn was necessary for optimal recovery. If the minicolumn elution solvent was methanol without acetic acid, recovery was only 52%. The use of methanol–acetic acid (98 + 2) did not improve recovery over that obtained with methanol–acetic acid (99 + 1). Overall recovery for 40 different meat samples spiked with 0.2 ppm CPA and carried through the entire extraction and cleanup procedure was 70.4 ± 14.1%. The procedure was linear over the range 0.016–15.6 ppm CPA (r^2 = 0.997) (Table 1).

Figure 2 is an LC chromatogram of a muscle sample (breast

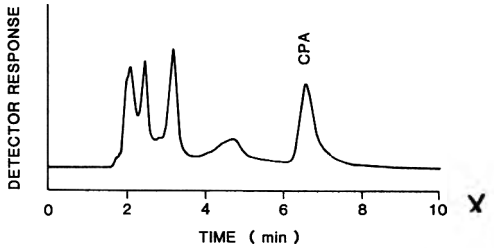

Figure 2. Liquid chromatogram of meat sample from chicken administered 10 mg CPA/kg body weight (via crop intubation) and killed 48 h later. Sample prepared for CPA analysis as described in text.

and thigh) prepared from a chicken killed 48 h after administration by crop intubation of 10 mg CPA/kg body weight. A sample from a bird administered solvent (1.0N $NaHCO_3$) only was also analyzed for CPA. Assuming a recovery of 70%, the amount of CPA in the muscle was 1.45 ppm, or 14.5% of the dose. By way of comparison, in our previous study of the distribution of [^{14}C]CPA in rats, 28% of the radioactivity was accounted for in skeletal muscle 48 h after oral administration of 5.0 mg/kg body weight (14). Whether the difference in apparent CPA content of the muscle is due to species differences or to presence of metabolites of CPA in the muscle has not been determined. We are currently investigating the occurrence of CPA in chicken meat after administration of varying amounts of CPA and at intervals after administration.

Acknowledgments

The authors thank Pamela Hayes and Philip Stancel for technical assistance.

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Cyclopiazonic Acid Production by Cultures of *Aspergillus* and *Penicillium* Species Isolated from Dried Beans, Corn Meal, Macaroni, and Pecans

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Ninety-five isolates of *Aspergillus* and *Penicillium* species from selected dried foods were examined for their ability to produce cyclopiazonic acid (CPA). The isolates were grown in sterile synthetic liquid medium at 28°C for 8 days in the dark. The medium and mold mycelia were then extracted with chloroform. CPA was semiquantitatively determined by thin layer chromatography through visual comparison with standards. The cultures of *A. flavus* were also examined for their ability to produce aflatoxin. One *A. tamarii* and all 13 *P. urticae* isolates produced CPA, whereas only 19 of the 31 (61%) *A. flavus* isolates produced CPA, and 6 (19%) *A. flavus* produced aflatoxin. All 13 *P. urticae* isolates also produced patulin and griseofulvin. CPA-producing *A. flavus* was found in all food types but not in all samples. CPA-producing *P. urticae* was found only in dried beans and macaroni.

Cyclopiazonic acid (CPA) is an indole tetramic acid produced by several species of *Aspergillus* and *Penicillium,* including *A. flavus, A. versicolor, A. oryzae, A. tamarii, P. patulum* (*P. urticae*), *P. cyclopium, P. viridicatum, P. puberulum, P. crustosum,* and *P. camemberti* (1–5). Some isolates of *A. flavus* produce both CPA and aflatoxin (6, 7). Both mycotoxins are mutagenic singly and in combination to *Salmonella typhimurium* TA 98 (8). CPA has been found as a natural contaminant of corn (6), peanuts (9), and cheese (10). Aflatoxins have been found in these same agricultural products. Contamination of foods and feed by these mycotoxins can lead to health problems in animals and can result in economic losses (11).

Received February 21, 1986. Accepted May 22, 1986.
[1] Division of Microbiology.

The purpose of this study was to evaluate which of the most frequently occurring *Penicillium* and *Aspergillus* species isolated from dried beans, corn meal, macaroni, and pecans produce CPA. The production of aflatoxins by selected *A. flavus* isolates and of patulin and griseofulvin by all *P. urticae* isolates was also investigated.

Experimental

Food Collection

A total of 150 dried food products (packaged foods): 30 each of navy beans, pinto beans, corn meal, macaroni, and pecans, were purchased from grocery stores in the Washington, DC, area. No more than 10 products were purchased at any one time.

Mold Flora Determination and Isolate Preparation

Each food was examined for the presence of viable mold according to a published method (12). Detectable species of *Aspergillus* (13), *Penicillium* (14), and other genera (15, 16) were identified.

Culture Conditions

Pure isolates of *Aspergillus* and *Penicillium* were grown on Czapek agar plates. After 7 days, the isolates were transferred to freshly prepared synthetic liquid medium (17). The medium contained the following (g/L): glucose 60, $NaNO_3$ 4.5, $MgSO_4 \cdot 7H_2O$ 0.5, KCl 0.5, and K_2HPO_4 1.0. The medium was supplemented by 0.5 mL/L of a trace metal solution containing (g/L): $Na_2B_4O_7 \cdot 10H_2O$ 1.4, $(NH_4)6Mo_7O_{24} \cdot 4H_2O$

1.0, $CuSO_4 \cdot 5H_2O$ 0.6, $MnSO_4 \cdot H_2O$ 0.22, $ZnSO_4 \cdot 7H_2O$ 35.2, and $FeSO_4 \cdot 7H_2O$ 20. The ingredients were mixed and the pH was adjusted to 5.5 with 2N HCl. Portions of the medium, 100 mL, were poured into 500 mL Erlenmeyer flasks, sealed with cotton plugs, and autoclaved for 20 min at 120°C and 15 psi. After cooling, the flasks were inoculated with spores of selected mold species and were kept stationary in the dark at 28°C for 8 days. An isolate (NRRL 3251) of *A. flavus* known to produce CPA and aflatoxin and an isolate (NRRL 6388) of *A. flavus* known to produce CPA but not aflatoxin (J. A. Lansden, National Peanut Research Laboratory, Dawson, GA, personal communication, 1985) were also cultured under these conditions as controls to confirm production of these toxins.

Extraction

After incubation, a long-stem funnel was placed in the flask through the cotton plug and 100 mL chloroform was added. The culture broth and the mycelial mat were extracted together by soaking and occasionally shaking for 24 h. The mixture was heated in a steam bath until it boiled. After cooling, 10 mL of the chloroform layer was withdrawn and filtered through 5 g anhydrous sodium sulfate. The filtrate was collected in a 4 dram vial. The sodium sulfate was washed with 2 mL chloroform and was collected in the same vial. The extract was evaporated to dryness on a steam bath under a stream of nitrogen. All extracts were analyzed for CPA, and extracts of *A. flavus* were also analyzed for aflatoxin; extracts of *P. urticae* were also analyzed for patulin and griseofulvin.

Thin Layer Chromatography

CPA and aflatoxin were separated by thin layer chromatography (TLC) on silica gel 60 precoated glass plates (Merck 5763).

Determination of CPA.—The TLC plates were dipped in 2% alcoholic oxalic acid solution and dried at 80°C for 1 h. The residue was dissolved in 200 μL chloroform, and 2 and 5 μL aliquots of the chloroform solution were applied on the plate along with 2, 4, 6, and 10 μL CPA standards (100 ng/μL chloroform). The plate was developed with benzene–acetic acid–methanol (90 + 5 + 7) and then dried at room temperature. CPA was visualized by spraying with 1% *p*-dimethylaminobenzaldehyde in 75 mL ethanol and 25 mL HCl (18). The color was allowed to develop for 10 min at room temperature. CPA appeared as a bluish purple spot with R_f 0.60. Color intensity of the sample spots was visually compared with that of the standards.

Determination of aflatoxin.—The TLC procedure from *Official Methods of Analysis* (secs. **26.011-26.012**, 19) was used. The mobile phase was ethyl ether (anhydrous)–methanol–water (96 + 3 + 1).

Determination of patulin and griseofulvin.—The mobile phase was toluene–ethyl acetate–90% formic acid (5 + 4 + 1). After development, the plate was examined under long-wave UV light. Griseofulvin appeared as a blue fluorescent spot with R_f ca 0.6. The plate was then sprayed with 0.5% 3-methyl-2-benzothiazolinone hydrazone aqueous solution and heated at 130°C according to sec. **26.131** (18). Patulin appeared as a yellow-brown fluorescent spot at R_f ca 0.5.

Confirmation

The identity of CPA in 2 of the positive samples was confirmed by mass spectrometry/mass spectrometry as follows. A portion of the sample extract, calculated to contain ≥200 ng CPA, was applied as a single spot on an oxalic acid-impregnated TLC plate, and spots of standard CPA were applied at both sides of the same plate. After development, the central portion over the sample was covered with a glass plate, and the standards were sprayed with the 1% *p*-dimethylaminobenzaldehyde solution for visualization. The area of the sample having the same R_f as the standards was scraped. The silica gel was collected on a piece of Al foil and was transferred to a 10 mL syringe with a male Luer outlet fitted to a 0.5 μm PTFE Millex-SR filter unit (SLS RO 25 NS, Millipore Corp., Bedford, MA 01730). CPA was eluted into a 2 dram vial with eight 0.6 mL portions of chloroform–acetone–isopropanol (7 + 1.5 + 1.5). The solvent was evaporated, and the residue was dissolved in chloroform.

Samples and standards were analyzed under positive ion chemical ionization conditions (0.22 torr CH_4, source temperature 250°C, 0.30 mA emission, 70 eV electron energy). Samples were introduced into the Finnigan TSQ-46 quadrupole mass spectrometer by direct probe using ballistic heat-

Table 1. Mold species detected in 150 dried food products

Species	No. samples positive
Alternaria spp.	27
Aspergillus candidus	49
A. clavatus	8
A. flavus	98
A. fumigatus	6
A. glaucus	149
A. nidulans	3
A. niger	45
A. ochraceus	35
A. restrictus	22
A. sydowi	7
A. tamarii	15
A. terreus	44
A. ustus	1
A. versicolor	23
A. wentii	11
Botrytis cinerea	2
Cephalosporium sp.	1
Cladosporium herbarum	51
Fusarium spp.	21
F. moniliforme	20
Mucor spp.	41
Paecilomyces varioti	2
Penicillium brevi-compactum	15
P. chrysogenum	7
P. citrinum	28
P. commune	3
P. corylophilum	2
P. cyclopium	60
P. expansum	2
P. frequentans	10
P. islandicum	10
P. lanosum	3
P. luteum	1
P. martensii	1
P. meleagrinum	1
P. oxalicum	20
P. palitans	7
P. purpurogenum	2
P. rubrum	5
P. rugulosum	1
P. thomii	1
P. urticae	32
P. variabile	2
P. viridicatum	44
Rhizopus nigricans	61
Scopulariopsis spp.	5
Stemphylium sp.	1
Syncephalastrum spp.	24
Trichoderma viride	5
Trichothecium roseum	3

Table 2. CPA production in liquid medium at 28°C from *Penicillium* species

Species	Source	No. isolates investigated	No. producing CPA (μg/mL)
P. brevi-compactum	navy beans	1	ND[a]
P. cyclopium	pinto beans	3	ND
	navy beans	8	ND
	corn meal	4	ND
P. islandicum	macaroni	1	ND
P. martensii	navy beans	1	ND
	corn meal	2	ND
P. urticae	navy beans	2	2 (6, 8)
	pinto beans	1	1 (4)
	macaroni	10	10 (4–10)
P. variabile	corn meal	1	ND
P. viridicatum	pinto beans	6	ND
	corn meal	2	ND

[a] Not detected (<100 ng/mL).

Table 3. Aflatoxin and CPA production in liquid medium at 28°C from *Aspergillus* species

Species	Source	No. isolates investigated	No. producing: Aflatoxin (ng/mL)	No. producing: CPA (μg/mL)
A. candidus	corn meal	1	ND[a]	ND[b]
	pinto beans	1	ND	ND
A. clavatus	corn meal	2	ND	ND
A. flavus	pinto beans	6	ND	6 (0.2–15)
	navy beans	3	1 (1000)	1 (2)
	corn meal	8	3 (4–10 000)	5 (0.1–8)
	macaroni	11	ND	4 (4–10)
	pecans	3	2 (5–200)	3 (4–10)
A. flavus	NRRL 6388	4	ND	4 (8–20)
A. flavus	NRRL 3251	4	4 (500–2000)	4 (5–10)
A. ochraceus	navy beans	1	ND	ND
	pinto beans	3	ND	ND
A. tamarii	macaroni	1	ND	1 (10)
A. terreus	corn meal	3	ND	ND
	macaroni	7	ND	ND
A. ustus	corn meal	1	ND	ND
A. versicolor	pinto beans	1	ND	ND
A. wentii	navy beans	1	ND	ND

[a] Not detected (<1 ng/mL).
[b] Not detected (<100 ng/mL).

ing. The collision-activated decomposition spectrum of the $(m + H)^+$ ion at m/z 337 was obtained at 15 eV collision energy. Relatively abundant fragments at m/z 196 and 182 and less abundant ions at m/z 154, 140, and 98 were observed. Spectra obtained from samples and standards were essentially identical.

The identity of aflatoxin in all products was confirmed by preparing the aflatoxin–water adduct, using trifluoroacetic acid as a catalyst according to sec. **26.083** (19).

The identities of patulin and griseofulvin were supported by spotting samples along with internal standards. Samples were considered to be positive when sample toxin spots and internal standards appeared as a single spot after development on a TLC plate.

Results and Discussion

Table 1 lists the mold species and genera detected in the 150 dried food products. Fifteen genera and at least 51 species were detected, most of the species being in the genera *Aspergillus* and *Penicillium*. *A. glaucus* (149 products) and *A. flavus* (98) were detected in more than one-half of the products, and *P. cyclopium* (60), *Rhizopus nigricans* (61), and *Cladosporium herbarum* (51) were detected in more than one-third. The remaining organisms were less frequently encountered. Although not indicated in Table 1, with the exception of *A. glaucus*, certain of the mold species were more prevalent in one food type than in another. For instance, *A. ochraceus* was detected only in dried beans, more than half of which also contained *P. cyclopium* and *P. viridicatum*. *Fusarium moniliforme* and *P. oxalicum* were detected only in corn meal. All 30 elbow macaroni products contained *A. flavus*, *A. candidus*, and *A. terreus*, and 27 of them contained *P. urticae*. The chief molds in pecans were *A. flavus* and *A. niger*. These results suggest that certain mold species may prefer one food type over another, whereas other species, e.g., *A. glaucus*, are nonspecific.

Table 2 lists CPA results obtained with the 42 *Penicillium* isolates screened in this study. Of the 7 species investigated, only the 13 isolates of *P. urticae* were positive. In addition, these 13 isolates also produced patulin and griseofulvin. In an earlier study with dried beans, 9 of 9 isolates of *P. urticae* were positive for patulin and griseofulvin (20). Apparently most isolates of *P. urticae* produce these toxins (in relatively high amounts) and possibly also CPA. Thus, *P. urticae* may be a more important toxigenic fungus than has been considered to date. Its high incidence rate in macaroni suggests that this food type be investigated for the natural presence of these 3 toxins.

The negative CPA results for *P. cyclopium* were unexpected because this species has long been known to be a CPA producer (21). However, another study found that of 20 randomly selected *P. cyclopium* isolates examined, only 2 produced CPA (22). Of the 5 CPA-negative species, only *P. viridicatum* has been reported previously as a CPA producer (5).

Table 3 lists CPA results obtained with the 53 *Aspergillus* isolates screened. Of the 9 species investigated in this study, only the one isolate of *A. tamarii* and 19 of 31 isolates of *A. flavus* were CPA-positive. Only one of the 7 CPA-negative *Aspergillus* species, *A. versicolor*, has a previous history of CPA production (5). Only 6 of the 31 *A. flavus* isolates produced aflatoxin; all 6 were also positive for *A. flavus* (NRRL 6388) CPA. Aflatoxin alone was never detected. The control isolate *A. flavus* (NRRL 3251) produced CPA and aflatoxin, while *A. flavus* (NRRL 6388) produced CPA. These results indicate that CPA may be a more common metabolite of *A. flavus* than is aflatoxin, and thus is also likely to be present in aflatoxin-contaminated foodstuffs.

In conclusion, finding CPA-producing mold isolates in all 5 food types examined would seem to justify (*1*) the development of analytical methods for the detection of this metabolite in these food types and (*2*) the investigation of other types of foods, including cottonseed meal and other nuts, for the presence and prevalence of CPA-producing molds and for CPA itself.

Acknowledgments

We thank John A. Lansden (U.S. Department of Agriculture, National Peanut Research Laboratory, Dawson, GA) for providing the CPA standard and the fungal strains (NRRL 3251 and NRRL 6388). We thank William C. Brumley (Food and Drug Administration) for obtaining the CPA mass spectra.

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Isolation and Liquid Chromatographic Determination of the Cyclic Peptide Mycotoxin Cyclosporin A from Rice

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A simple method for determination and quantitation of a cyclic peptide mycotoxin, cyclosporin A, in rice is presented. Rice inoculated with *Trichoderma polysporum* (Link ex Pers.) was extracted with methylene chloride after 4 weeks of incubation. Cyclosporin A was isolated from extracts by using open bed gel filtration column chromatography (LH-20, acetonitrile) and monitored with thin layer chromatography and reverse phase liquid chromatography coelution with a standard. Preliminary thin layer chromatographic methods were developed. Cyclosporin A was detected by iodine and after partial acid hydrolysis by ninhydrin and UV light. A liquid chromatographic method was developed that used a reverse phase disposable cartridge cleanup and isocratic chromatography with a reverse phase octadecylsilica column and a UV detector set at 212 nm. Recovery of cyclosporin A from spiked rice samples (mg/g range) was 85%.

Toxic peptides as microbially derived food contaminants have been relatively unrecognized as potential health hazards until recent years (1–3). Although cyclic peptide phallotoxins and amatoxins have been known for many years to be the causative agents in mushroom poisoning (2), recent studies have identified other cyclic peptide toxins of bacterial and fungal origin in soil, food, and livestock feed (3–4). Cyclochlorotine, a cyclic oligopeptide, has been isolated from *Penicillium islandicum*, a fungus commonly found on yellowed rice (1). Cyanoginsin-LA, a cyclic heptapeptide, has been characterized as the toxic principle of a cyanobacterium causing algal poisoning in cattle (3). Leucinostatins are peptide mycotoxins that are produced by *Paecilomyces silacimus* and cause liver damage in test animals (4).

The cyclic peptide cyclosporin A, a metabolite of *Trichoderma polysporum* (Link ex Pers.), has been intensively studied because of its exceptional immunosuppressive properties (5). Cyclosporin A (Figure 1) is a cyclic undecapeptide containing 11 amino acids, all of which are of the naturally occurring S configuration with the exception of D-alanine (6). The compound has 7 *N*-methylated amino acids, one of which is a unique amino acid with an olefinic side chain (position 1). Cyclosporin A was initially isolated from the culture broths of *T. polysporum* and used as a postoperative immunosuppressive agent. It is similar to other mycotoxin peptides both in its diverse range of biological activities (hepato- and nephro-toxicity) and its physical properties (7). Several liquid chromatographic methods have been reported for the analysis of cyclosporin in both human serum and urine (8–11). However, little has been reported on the separation and analysis of the compound and other cyclic peptide mycotoxins from food and feed sources.

The present study involves development of techniques for quantitative assessment of cyclosporin A in rice. The methods outline open-bed column, thin layer, and liquid chromatographic procedures for the separation and quantitative determination of cyclosporin A from *T. polysporum*-inoculated rice.

Received February 24, 1986. Accepted May 31, 1986.

METHOD

Apparatus

(a) *Densitometer.*—Schoeffel SLD (Schoeffel Instrument Div., 24 Brooker St, Westwood, NJ 07675). Scan within lower wavelength UV region.

(b) *Liquid chromatograph.*—Waters Associates (34 Maple St, Milford, MA 01757) M01757 equipped with Digital Professional 350 System interface, 510 pump, WISP 710 B injector, and LA 50 Digital 50 printer.

(c) *Chromatographic columns.—Liquid chromatography.*—Versapack C-18, 10 μm (Alltech Associates, 2051 Waukegan Rd, Deerfield, IL 60015), 4.1 mm id × 25 cm long. *Open-bed gel permeation chromatography.*—Sephadex LH-20 (Pharmacia, Box 175, Uppsala, S-75104 Sweden), 2 × 57 cm.

(d) *Detectors.*—Waters Model 441 ultraviolet detector operated at 212 nm for absorbance of amide bonds. Beckman DU-88 spectrophotometer (Beckman Scientific Instr. Div.,

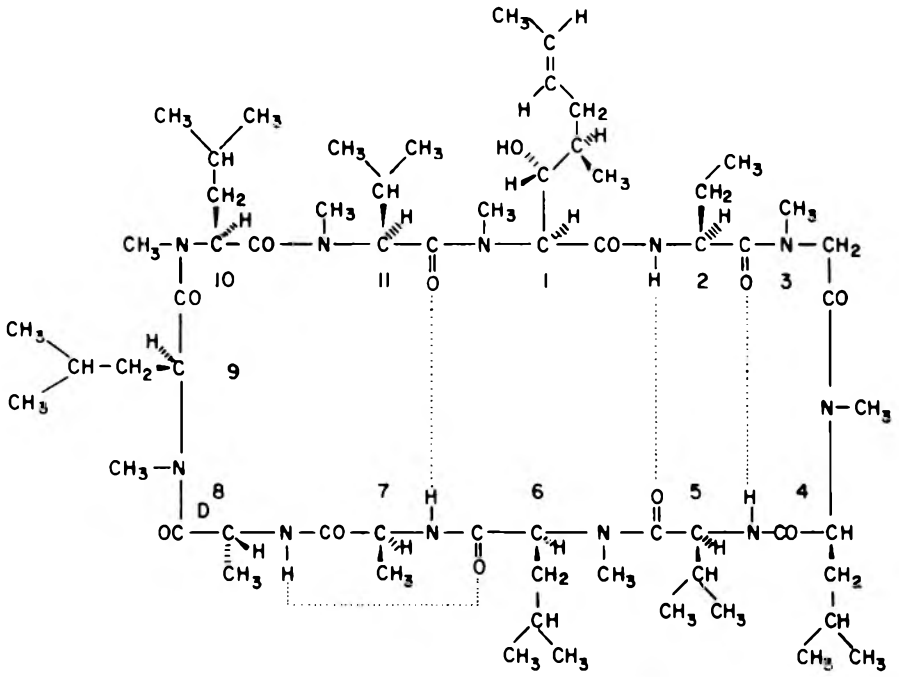

Figure 1. Structure of cyclosporin A.

Campus Dr, Jamboree Blvd, Irvine, CA 92713), λ Scan III high performance module for scanning column eluant. Infrared spectrophotometer (Beckman Aculab 1).

(e) *Thin layer chromatographic plates.*—Glass plates (10 cm) precoated (fluorescent) with 0.25 mm silica gel 60 (EM Science, 2909 Highland Ave, Cincinnati, OH 45212).

(f) *Iodine chamber.*—Glass cabinet 8 × 28 × 28 cm, bottom surface covered with iodine pellets.

(g) *Disposable cartridges.*—Sep-Pak RP-18 silica cartridges (Waters Associates).

Reagents

(a) *Solvents.*—Liquid chromatographic grade acetonitrile, reagent grade methylene chloride, chloroform, *n*-butanol, methanol, ethyl acetate, and hexane. Water purified by Milli-Q system (Millipore Corp., 80 Ashby Rd, Bedford, MA 01730). The following aqueous solutions were prepared: saturated sodium bicarbonate solution, saturated sodium chloride solution, and 1N HCl.

(b) *Standard.*—Pure cyclosporin A was obtained from Hyun Lillehoj (U.S. Department of Agriculture, Beltsville, MD). Purity had previously been established through fast atom bombardment mass spectrometry and substantiated through observation of a single spot on 3 different TLC solvent systems, a single peak in reverse phase liquid chromatographic separation on 3 different LC systems (8–10), and IR (not shown here) (12).

Production of Cyclosporin A

T. polysporum was obtained from K. J. Kwan-Chung (National Institutes of Health, Bethesda, MD). Inoculum was prepared from conidia produced on nutrient agar slants. Autoclaved, converted rice (40% moisture) was inoculated with spores from single slant per 300 g substrate in 2.8 L Fernbach flask with subsequent static incubation for 4 weeks at 28°C. Moldy rice from single flask was extracted by suspension in 1 L methylene chloride and subsequently ground in Waring Blendor. Extract was filtered and solvent was removed by vacuum evaporation.

Sample Preparation from Rice

In 300 mL, round-bottom flask, extract nonpolar lipids from methylene chloride-derived material by vigorous stirring with 200 mL *n*-hexane for 1 h at room temperature. Filter through sintered glass filter funnel and aspirate hexane into filter flask. Stir residual solids with two 200 mL portions of ethyl acetate and remove solvent by vacuum filtration. Evaporate ethyl acetate extract in 500 mL round-bottom

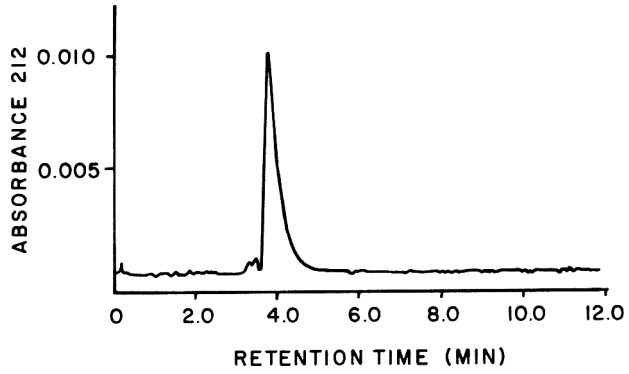

Figure 2. Liquid chromatogram of cyclosporin A.

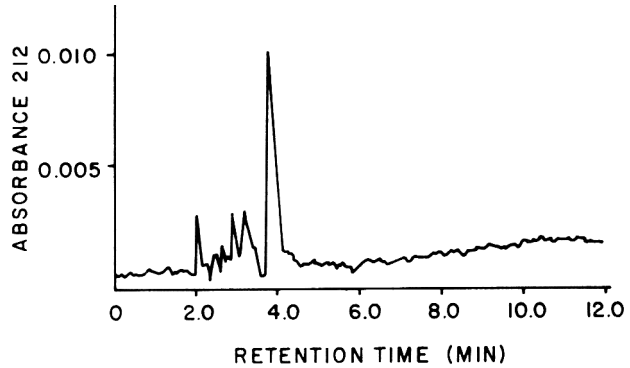

Figure 3. Liquid chromatogram of cyclosporin A recovered from rice.

flask on rotary evaporator. Combine extracted residue with water (300 mL) and use a 500 mL separatory funnel to extract aqueous suspension with three 150 mL portions of methylene chloride. Add saturated NaCl for controlling emulsion-free separation. Combine methylene chloride extracts. Wash twice each with 60 mL aqueous solutions of saturated sodium bicarbonate, 1N HCl, and saturated sodium chloride and dry over 3 g sodium sulfate. Evaporate methylene chloride in rotary evaporator to yield 250 mg of light brownish residue.

Chromatographic Separation of Cyclosporin A

(a) *Open-bed chromatography.*—Dissolve 80 mg residue from preliminary purification in minimal volume of acetonitrile (1–2 mL). Apply sample solution to LH-20 column and elute column with acetonitrile. Collect 5.5 mL fractions at flow rate of ca 8 mL/h. Analyze fractions by TLC, LC coelution with standard (Figure 2), and IR measurement (an intense band at 1630–1670/cm indicative of the peptide amide bond [12]). Pool cyclosporin A fractions (23–27; requiring a volume of 210–250 mL) and remove acetonitrile by evaporation under vacuum (rotary evaporator using water aspirator at 50°C), leaving 5 mg clear oil identical on TLC, IR, and UV analysis to cyclosporin A standard.

(b) *Liquid chromatography.*—Dissolve 5 mg residue from preliminary purification in 0.2 mL acetonitrile–water mixture (8 + 2, v/v). Wash disposable cleanup cartridge with acetonitrile (2 mL) followed by water (2 mL) and final 2 mL acetonitrile–water solution (1 + 1, v/v). Apply sample to cleanup cartridge and elute with 4 mL acetonitrile–water (3 + 2, v/v) into vial. Dilute eluted sample to 5 mL and place in automatic sample injection system. Inject 0.10 mL in C-18 Versapack column with acetonitrile–water as mobile phase and flow rate of 1 mL/min (Figure 3).

(c) *TLC determination of cyclosporin A.*—Dissolve rice-extracted sample (~2 mg) in methanol (2 mL) and apply 5–25 μL volumes to silica gel plate. Develop TLC plates in 3 solvent systems: (*1*) *n*-butanol–acetic acid–water (4 + 1 + 1, v/v/v), (*2*) chloroform–acetic acid–methanol (85 + 10 + 5, v/v/v), and (*3*) ethyl acetate–hexane–acetone (2 + 1 + 1, v/v/v) in equilibrated developing tanks. Dry TLC plates with hot-air blow gun and place in iodine chamber for 15 min to determine iodine-reactive substances. Sublime iodine from plates by placing in oven at 110°C for 15 min. Spray plate with 6N HCl and oven-dry at 110°C for 30 min. Spray dried plates with 0.1% ninhydrin solution in *n*-butanol. An orange to brown spot in samples with same R_f as standard spot is considered positive confirmation for cyclosporin A. R_f values for cyclosporin A were: solvent *1*, 0.83; solvent *2*, 0.92; solvent *3*, 0.81.

Cyclosporin A Quantitation

Dissolve pure cyclosporin A (50 mg) in 10 mL acetonitrile–water (8 + 2, v/v) and prepare series of standard solutions (1 + 2, 1 + 3, 1 + 4, 1 + 5, 1 + 8, 1 + 10) by serial dilution of 1 mL aliquots of standard solution. Load automatic sample injection system with standard solutions, inject, and then elute with acetonitrile–water system (80 + 20, v/v). Plot standard curve (concentration vs area) and use it for determinations of cyclosporin A.

Confirmatory Test

Analyze cyclosporin A samples by obtaining UV and IR spectra. Obtain IR spectra by preparing KBr pellets; obtain UV spectra through dissolution of cyclosporin A samples in methylene chloride. Positive confirmation of cyclosporin A is indicated if sample IR spectrum is identical with standard and in complete agreement with reported (12) spectrum (1630–1670, amide; 970, CH = CH *trans*). Likewise, UV is characterized by λmax of 200 nm.

Table 1. Recovery (%) and lowest detectable levels (μg/kg) of cyclosporin A added to rice

% Recovery	Amount cyclosporin A added, μg/kg × 10^2	Lowest detectable amount, μg/kg × 10^2
84.6	2.7	2.3
84.9	3.7	3.1
85.1	13.7	11.7
84.7	25.5	21.6
85.3	38.8	33.6
86.2	51.0	43.0
Av. 85.1		
SD[a] 0.60		

[a] Triplicate samples were analyzed at each spiking level.

Assessment of Recoveries

Add weighed quantity (1–4 mg range) of cyclosporin A to rice (100 g) and shake 30 min. After allowing to stand overnight, mix rice with methylene chloride (250 mL). Filter sample extracts on Buchner funnel and extract spiked samples by methods described for extraction of fungal-contaminated rice. Dissolve 20 mg sample of residual oil in 32 mL acetonitrile–water (80 + 20, v/v). Elute solution aliquot (2 mL) on disposable cleanup cartridge prewashed with 2 mL acetonitrile followed by 2 mL water and 2 mL acetonitrile–water (50 + 50, v/v). Prepare 4 sample dilutions, elute on LC column, and quantitate peaks on the basis of the calibration curve.

Results and Discussion

The cyclic peptide mycotoxin cyclosporin A from extracted rice samples was quantitated following cleanup by hexane and acid-base aqueous extraction of the initial methylene chloride extract. Sep-Pak cleanup and analysis on a C-18 Versapack column using an acetonitrile–water (8 + 2, v/v) isocratic mobile phase provided a simple routine method for chromatography of cyclosporin A. The retention time was 4.1 min. The amount of cyclosporin A was calculated from a standard calibration curve (sample concentration vs peak area) which was plotted within a 0.5–12 × 10^2 μg range. Gel filtration, adsorption chromatography with an LH-20 (Sephadex) column, and elution with acetonitrile were used for a preparative scale purification of cyclosporin A. Yields ranged from 0.040 to 0.10 mg cyclosporin A/g rice (13).

Rice samples were spiked with cyclosporin A and extracted to determine the efficacy of the extraction system. Milligram quantities of cyclosporin A were added to g quantities of rice. The rice samples were subsequently extracted with methylene chloride, and the extracts were chromatographically processed for determination of cyclosporin A. Independent determinations of smaller quantities (less than 1 mg) were made, suggesting the methods employed are amenable to analysis of quantities in the ppm range. Blank chromatographic determinations of rice free of cyclosporin A revealed no extraction artifacts eluting at or near its retention time that would interfere with its quantitation. The linearity (r^2) of the cyclosporin A quantitation calibration was 0.99 within the 0.5–12 × 10^2 μg range. As shown in Table 1, recovery in replicated tests averaged 85% for premixed samples containing cyclosporin A within the range detected in the inoculated rice samples.

Because of the solubility of cyclosporin A in organic solvents routinely used in acid and base work-ups and the absence of acidic and basic functionalities in cyclosporin A, the method provides for removal of ionizable acids and bases which might interfere with the determination. This may be adapted in similar determinations of other known cyclic peptide mycotoxins that contain no ionizable functionalities.

Although previous reports (8, 9) of chromatographic separation of cyclosporin from blood serum cite the necessity for relatively high column temperatures (72°C), the good baseline resolution at ambient conditions demonstrates no necessity for temperatures above ambient. No improvement was observed with the addition of methanol to the chromatographic solvent system. The combination of Sep-Pak cleanup, liquid chromatography, and UV detection provides a rapid, simple approach for quantitative detection of cyclosporin A in a cereal grain commodity.

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Fast and Sensitive Screening Method for Detection of Trichothecenes in Maize by Using Protein Synthesis Inhibition in Cultured Fibroblasts

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A fast and sensitive bioassay with hamster (BHK-21 C13) fibroblasts for the detection of toxic trichothecenes in maize is described. Cells are exposed to pure toxins or crude maize extracts for 30 min. The mixture is then incubated with [1-^{14}C]-leucine for an additional 60 min and the radioactivity incorporated into the protein of the washed cells is determined. The sensitivity of the assay was in the range 1-10 ng/mL (or 50 ppb in maize) for T-2, HT-2, and diacetoxyscirpenol. At least 1000-fold higher concentrations of non-trichothecene mycotoxins and plant toxins were necessary to cause an inhibition of protein synthesis in the cells. Of 24 maize samples tested, 14 gave a positive response in this assay and the presence of trichothecenes could be confirmed chemically in 11 samples. Therefore, the described bioassay is proposed as a useful screening method for cytotoxic trichothecenes in maize.

Fusarium molds are known as producers of several toxic secondary metabolites which can contaminate cereal grains in the field or in storage. Among these mycotoxins are the structurally highly diverse group of trichothecenes, frequently associated with fusariotoxicosis in farm animals and humans (1, 2). A whole variety of analytical methods based on highly efficient separation techniques such as thin layer chromatography (TLC), gas chromatography combined with mass spectrometry (3), or liquid chromatography (4) are presently available for the detection and quantitation of particular trichothecenes. In addition, immunological methods—radioimmunoassays and enzyme-linked immunospecific assays (5)—have been developed for the most frequently found trichothecenes. However, some reasons favor the application of a biological method: Trichothecenes are a group of highly cytotoxic compounds variable in structure but with a common mode of action (6). A bioassay based on specific toxicity could be suitable as a screening method for the detection of toxicologically relevant trichothecenes in feed. In the following, we report on our investigations using the inhibition of protein synthesis in cell cultures as a measure for the quantitative determination of trichothecenes.

Received December 18, 1985. Accepted May 28, 1986.

METHOD

Apparatus and Reagents

(a) *Apparatus for cell culture.*—CO_2 incubator (Forma Scientific, Brouwer, CH-Luzern); Coulter Counter (Model ZBI, Coulter Electronics, Herts, England, IG, CH-Zürich); inverted microscope (Nikon, CH-Küsnacht); pipetting aid (O. Kleiner AG, CH-Wohlen); 6-well sterile tissue culture plates (Costar Cluster 6, Tecnomara AG, CH-Zürich); sterile tissue culture dishes (Nunc, Gibco, CH-Basel); sterile tubes (Falcon 2095, Becton Dickinson, CH-Münchenstein); unsterile tubes (Sarsted, CH-Sevelen); sterile transfer pipets and plastic pipet tips (Treff AG, CH-Degersheim); centrifuge (Beckmann, CH-Basel); liquid scintillation spectrometer (Betaszint 5000/300).

(b) *Toxins.*—T-2, HT-2, diacetoxyscirpenol (DAS), verrucarin, aflatoxin B_1, luteoskyrin, and emodin (Senn Chemicals, CH-Dielsdorf); vomitoxin (The Myco-lab Co., Chesterfield, MO); tetrahydroxyanthraquinone (Sigma Chemical Co.); ricinus lectin (RCA 60; RCA II) (Calbiochem AG, CH-Luzern). Dissolve mycotoxins in methanol and store at −20°C. Just before use, dilute toxins to be tested in F-12 medium (to which cells are exposed).

(c) *Maize samples.*—Maize kernel samples from Switzerland and abroad were collected between August and October

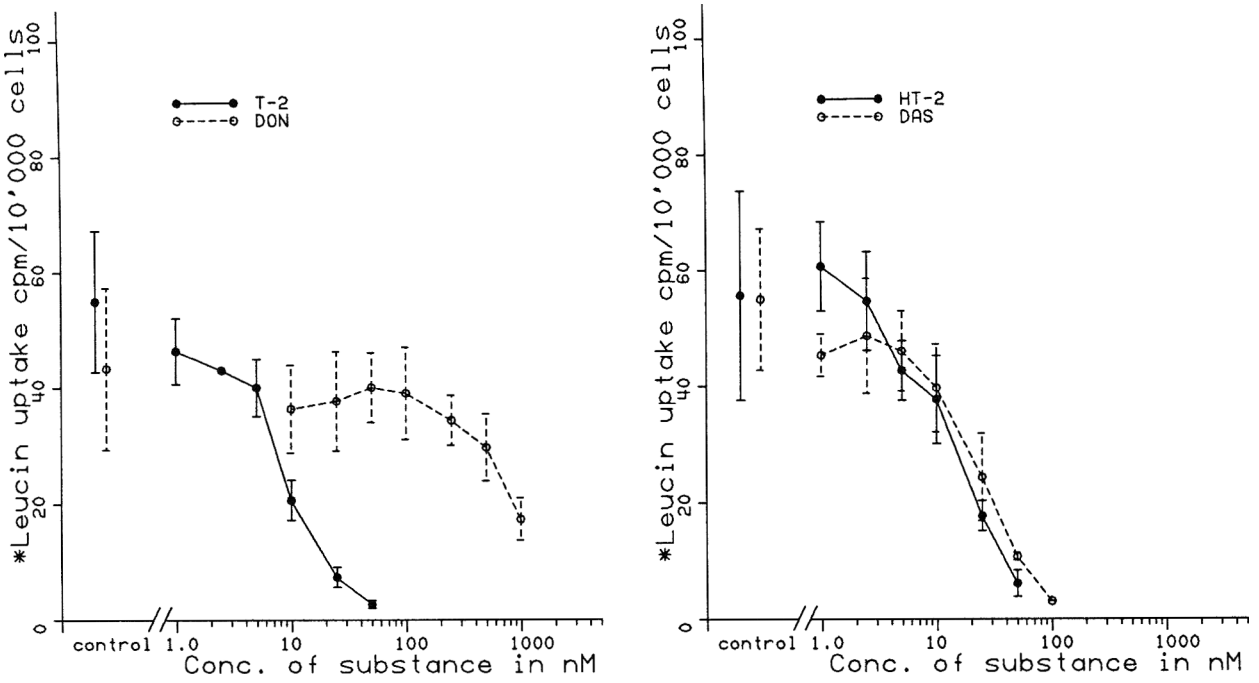

Figure 1. Typical experiments showing concentration-related effects of T-2 and DON (left), HT-2 and DAS (right) on protein synthesis activity in BHK fibroblasts.

1983, according to International Cereals Chemistry Standards (ICC No. 101).

Cell Culture

Culture Baby Hamster Kidney fibroblast line (BHK, American Type Culture Collection ATCC CCL 10) in Eagle's minimum essential medium (MEM) and F-12 medium supplemented with 10% fetal calf serum and antibiotics (streptomycin and penicillin: 50 000 IU/mL). Prepare trypsin as 0.25% (w/v) stock in sterile phosphate buffered saline (PBS) without Ca^{2+} and Mg^{2+} and store frozen until used. (All culture material available from Boehringer Mannheim.) Wash cells with Iso-Osmol (Wüst AG, CH-Schwerzenbach).

Maintain BHK cells by subculturing with trypsin (0.25% in PBS) or by storing in liquid nitrogen in MEM medium containing 10% dimethylsulfoxide and 10% fetal calf serum. Incubate cells in CO_2 incubator at 37°C and 5% CO_2.

Preparation of Cells

Prepare fibroblast suspension from confluent cultures in 15 cm culture dishes by washing with PBS followed by incubating cells under normal culture conditions with PBS containing 0.25% trypsin until cells begin to detach. Flush cells from dish with fresh MEM. Count number of cells per mL in suspension in Coulter Counter and transfer diluted cell suspension to 6-well plates at concentration of 25 000 cells/sq. cm. Let cells attach 24 h before assay is performed.

Preparation of Maize Extracts

Weigh 100 g of the maize sample into a 500 mL glass-stopper flask, add 200 mL methanol and 20 mL water, stopper, and shake 15 min using wrist-action shaker set at fast rate. Add 60 mL water and repeat shaking another 10 min. Decant through filter paper in 250 mL graduated cylinder and transfer 140 mL filtrate to 500 mL separatory funnel. Add 40 mL 25% NaCl solution and 180 mL CH_2Cl_2 and shake 1 min. Dry CH_2Cl_2 phase with Na_2SO_4. Filter into round-bottom flask and wash Na_2SO_4 with CH_2Cl_2. Evaporate to dryness. Dissolve residue in 0.5 mL methanol. Take 5 μL (corresponding to 0.5 g original sample) in 3 mL F-12 medium and dilute further, in steps of 1:10, for protein synthesis inhibition assay.

Chemical Identification of Fusarium Toxins

Purify extracts with Sep-Pak Florisil cartridges (Waters Associates, Inc.). To identify toxins, apply fractions on TLC plate and remove nonpolar impurities first with ether. Then develop in opposite direction with ether–ethanol (9 + 1). Spray with acidic *p*-anisaldehyde reagent.

Assay Procedure

Dissolve trichothecenes, aflatoxin B_1, anthraquinones, or crude maize extracts in F-12 medium. Remove MEM medium from 6-well plates and replace with 1 mL F-12 medium containing diluted sample. Expose cells 30 min to mycotoxins or to crude maize extracts at 37°C in incubator. At end of exposure time, add 0.05 μCi [1-^{14}C]-leucine (specific activity 429 μCi/mg, Amersham, H. W. Tschäppeler AG, CH-Zürich) per mL F-12 medium and incubate cells additional 60 min. Then remove medium, wash cells twice with Iso-Osmol, and detach cells with trypsin. Transfer cells to centrifuge tubes in 5 mL Iso-Osmol. Take 0.5 mL aliquot for cell counting and centrifuge 5 min at 1000 rpm. Remove Iso-Osmol supernate and place pellet in 10 mL Insta-gel (United Packard, Zürich). Determine amount of [1-^{14}C]-leucine incorporated into cells in liquid scintillation counter and plot as cpm/10 000 cells with appropriate data handling program (7) in computer.

Results and Discussion

All five fusaria toxins, T-2, HT-2, diacetoxyscirpenol (DAS), vomitoxin (deoxynivalenol, DON), and verrucarin A, had a concentration-dependent effect in our protein inhibition assay (Figure 1), whereas aflatoxin B_1 was ineffective even in much higher concentrations. For comparison, the plant toxins emodin, luteoskyrin, tetrahydroxyanthraquinone, and a ricinus lectin (RCA 60) were also tested. Our assay was very

Table 1. Effect of purified trichothecenes and other toxins on protein synthesis in BHK fibroblasts

Toxin	IC_{50},[a] ng/mL (±SD)
T-2 toxin	4 ± 0.6
HT-2 toxin	5 ± 1.4
Diacetoxyscirpenol (DAS)	10 ± 2.5
Verrucarin A	9 ± 4.3
Deoxynivalenol (DON)	209 ± 30.7
Luteoskyrin	4448[b] ± 861
Emodin	11 610[b] ± 1871
Tetrahydroxyanthraquinone	>7000[b]
Aflatoxin B_1	>5000
Agglutinin RCA 60 (RCA II)	>10 000

[a] IC_{50} values (concentration of toxin showing 50% inhibition of ^{14}C-leucine uptake in BHK fibroblasts) were graphically determined from 3–5 independent experiments and reported as mean ± standard deviation.
[b] Cytotoxic at higher concentration (cell detachment from culture disk).

insensitive for all these toxins except the trichothecenes (Table 1). The highest concentration of toxin or extract in F-12 medium contains 0.1% methanol, which has no effect on protein synthesis (unpublished data).

Our assay allows the detection of compounds that have a direct effect on protein synthesis. Aflatoxin B_1 did not depress protein synthesis because BHK cells show a relatively low activity of the microsomal mixed-function oxygenase system (8) that did not allow the activation of the toxin to the 8,9-epoxide within the short incubation time.

Anthraquinones are classified as inhibitors of energy production (9) and also of RNA synthesis (10, 11). Protein synthesis inhibition is only a consequence of the strong effect on RNA synthesis activity. The IC_{50} values and the high cytotoxicity of the 3 tested anthraquinones reflect these properties. The lectin RCA 60 inhibits protein synthesis after binding with an appropriate antibody (12). However, for protein synthesis inhibition to take place, the lectins may also bind to cell surface glycoproteins (13). Since in our bioassay RCA 60 shows no inhibition, BHK fibroblasts have presumably no such surface moieties.

Since trichothecenes are protein synthesis inhibitors (6), we studied the correlation between ^{14}C-leucine incorporation into the fraction of BHK cells precipitated by trichloroacetic acid and the complete ^{14}C-leucine uptake of washed whole cells during our bioassay. In both procedures, the assay showed the same sensitivity and therefore it seems appropriate to express the level of protein synthesis inhibition as ^{14}C-leucine uptake in the whole cells. Moreover, a laborious protein precipitation procedure, as described for example by Thompson and Wannemacher (14), is avoided and the time needed for this screening assay is therefore decreased.

It is important to note that the tested BHK-21 C13 cells were always in a logarithmic growth phase because we plated only 25 000 cells/sq. cm, which can grow up to 60 000 cells/sq. cm within 1 day. The assay was performed when about half of the full cell density was reached. There are conflicting reports on the sensitivity of different cell types used for trichothecene screening. On the one hand, Thompson and Wannemacher showed that T-2 toxin inhibited protein synthesis of 10 different epithelial and fibroblast cell lines (under confluent culture conditions) in almost identical concentrations. On the other hand, growth effects were markedly lower in slow growing cells such as human skin fibroblasts (15, 16). These reports may become less conflicting if we consider the fact that protein synthesis is more affected during logarithmic growth than during the stationary phase of confluency. Since 3T3 fibroblasts have been found to be very sensitive to trichothecenes when growth is measured (15), we compared this cell type with BHK cells in our assay. The sensitivity varied much more in 3T3 cells than in BHK cells, which is probably due to the fact that 3T3 cells have a relatively short but then very elevated logarithmic growth phase, followed by a very efficient contact inhibition of growth and protein synthesis at confluency.

Table 2. Effect of extracts from naturally infected maize kernels on protein synthesis in BHK cells

Sample	Inhibitory activity[a]	Determined T-2 units,[b] mg/kg	*Fusarium* toxins detected by TLC[c]
131	+	0.5	T-2
231	−	BDL[d]	none
232	−	BDL	none
233	−	BDL	none
331	−	BDL	none
431	+	0.05	HT-2, DON[e]
432	+	0.05	HT-2
433	−	BDL	none
434	+	0.5	T-2, DON,[e] DAS[e]
435	+	0.05	DON,[f] T-2[f]
436	−	BDL	none
437	−	BDL	none
438	+	0.05	HT-2
439	−	BDL	none
4310	−	BDL	none
4311	+	0.5	HT-2
4312	+	0.05	none
4313	+	0.05	none
532	+	0.05	T-2
533	+	0.05	HT-2
631	+	0.5	none
732	+	0.05	T-2
733	+	0.05	T-2,[e] DAS[e]
734	−	BDL	none

[a] Inhibitory activity found at maximum tolerated dose (0.17 g/mL F-12).
[b] T-2 units: amount of toxin calculated as T-2 (>0.05 mg/kg).
[c] T-2, HT-2, DAS, and DON.
[d] Below detection limit.
[e] GC (FID) confirmed.
[f] GC/MS confirmed.

The described method was used as a bioassay for the detection of trichothecenes in maize samples originating from various countries (Table 2). Aliquots of the crude methanol extracts were added to the cells and the protein synthesis inhibition assay was performed as described under *Method*. An inhibition of the protein synthesis was detected with 14 of 24 samples at the maximum tolerated concentration, corresponding to an amount of at least 0.05 mg T-2 toxin (or the toxicity-equivalents of other trichothecenes) per kg maize. The presence of trichothecenes (T-2, HT-2, or DON) could be confirmed chemically in 11 of these 14 cytotoxic samples, whereas none of those toxins were found in the 10 noncytotoxic samples. Therefore, the assay may be considered as a useful screening method for the detection of cytotoxic *Fusarium* toxins in maize. Compared to other screening systems with cell cultures using various growth assays for up to 14 days (14, 16–19), our assay is in the same range of sensitivity but less time consuming for all trichothecenes tested. Their specific effect on protein synthesis (6, 20) may make this system selective as a bioassay for trichothecenes even in the presence of other mycotoxins such as aflatoxins or plant toxins.

Acknowledgments

This work was supported by the Vereinigung Schweiz. Futtermittelfabrikanten and by the Schweiz. Kommission zur Förderung der Wissenschaftlichen Forschung.

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Mass Spectral Investigations on Trichothecene Mycotoxins. II. Detection and Quantitation of Macrocyclic Trichothecenes by Gas Chromatography/Negative Ion Chemical Ionization Mass Spectrometry[1]

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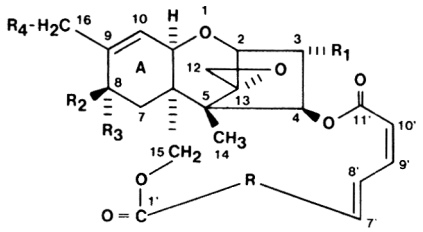
A general, sensitive gas chromatographic/negative ion chemical ionization mass spectrometric (GC/NICIMS) method of analysis was developed for the detection and quantitation of several polar, thermally labile, toxic macrocyclic trichothecenes. The procedure involves the conversion of the molecules to their corresponding alcohols (verrucarols) by alkaline hydrolysis, followed by derivatization of the hydrolysate with heptafluorobutyrylimidazole and analysis by GC/MS technique under negative ion chemical ionization conditions. Nanogram (250 ng) quantities of several macrocyclic trichothecenes with different verrucarol and ester moieties were analyzed successfully with good precision by this procedure. The method was applicable for the accurate determination of at least low ppb levels of these macrocyclic trichothecenes in environmental samples, such as fungal products, fermentation broths, and plant samples. This is the first reported, well developed, sensitive, and applicable method for the detection and quantitation of these compounds in naturally occurring samples.

Analysis for toxicants in the environment is a problem of wide interest. Although much of the effort in this area has been focused on synthetic chemicals, a number of naturally produced toxins pose serious hazards to humans and animals. One such class of compounds is the mycotoxins, which are fungal secondary metabolites that cause serious health problems for both humans and animals (1, 2). Within this class of toxins is a wide variety of chemical structures (3). Hence, the analytical chemist must use a large number of strategies for determining these compounds to prevent the economic loss and human health hazards caused by them.

One of the more important chemical groups within the mycotoxins is the trichothecenes (4). This group of closely related sesquiterpenoids is produced by various species of imperfect fungi including *Fusarium, Trichoderma, Myrothecium, Trichothecium,* and *Stachybotrys.* Because of their wide range and potency of biological activity, the trichothecenes are important natural products (4). The trichothecenes can be divided into 2 general groups: the simple trichothecenes [e.g., DAS (diacetoxyscirpenol) and T-2 toxin] and the macrocyclic trichothecenes [e.g., verrucarins (5), roridins (5), baccharinoids (6), satratoxins (7)]. The structures of the macrocyclic trichothecenes discussed in this report are shown in Figure 1.

A great deal has been published on analysis for simple trichothecenes principally because of their importance in agriculture (4, 8), although recently they have been implicated as chemical warfare agents (9, 10). The most sensitive and reliable method known for determining simple trichothecenes involves the conversion to their heptafluorobutyryl (HFB) esters followed by gas chromatographic/mass spectrometric (GC/MS) analysis under chemical ionization conditions and monitoring the negative ions (11, 12). Structurally similar, semisynthetic molecules such as deoxyverrucarol and 16-hydroxyverrucarol (Figure 2, A, F) have been found to be adequate internal standards both for detection and quantitation of simple trichothecenes (11). Recently, a

Received October 31, 1985. Accepted April 2, 1986.
[1] For Part I, see ref. 11.



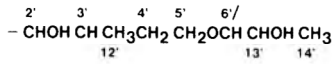
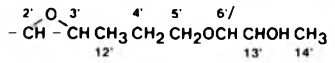
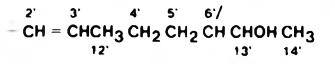
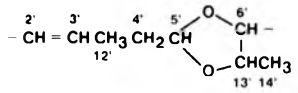
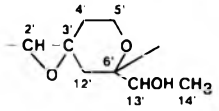
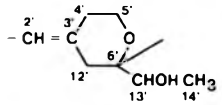
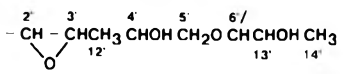
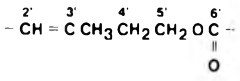
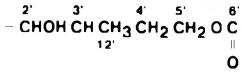
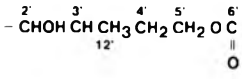
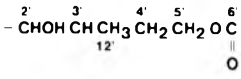
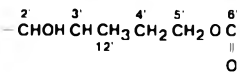
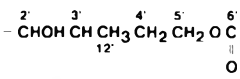
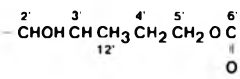
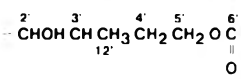
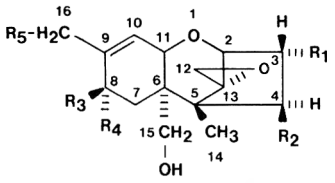
		R_1	R_2	R_3	R_4	OTHER
RORIDIN A (IA)		H	H	H	H	—
RORIDIN D (IB)		H	H	H	H	—
RORIDIN E (IC)		H	H	H	H	—
RORIDAN H (ID)		H	H	H	H	—
SATRATOXIN G (IE)		H	H	H	H	—
SATRATOXIN H (IF)		H	H	H	H	—
BACCHARINOL-4 (IG)		H	OH	H	H	—
VERRUCARIN J (IH)		H	H	H	H	—
VERRUCARIN A (II)		H	H	H	H	—
3α-HYDROXYVERRUCARIN A (IJ)		OH	H	H	H	—
8α-HYDROXYVERRUCARIN A (IK)		H	H	OH	H	—
8β-HYDROXYVERRUCARIN A (IL)		H	OH	H	H	—
16-HYDROXYVERRUCARIN A (IM)		H	H	H	OH	—
9,10-EPOXYVERRUCARIN A (IN)		H	H	H	H	9,10-EPOXY
8-KETOVERRUCARIN A (IO)		H	H	H	H	8-KETO

Figure 1. Structures for macrocyclic trichothecenes.

GC/MS method for determining the underivatized simple trichothecenes has been described in the literature (13). The compounds were introduced into the gas chromatograph via an on-column injector and analyzed by oxygen negative chemical ionization technique. The detection limits for some simple trichothecenes were determined to be 50–375 picograms (pg) by this method. A more direct MS/MS method also has been used for determining some of these molecules (14).

Although these techniques have proven very powerful for



COMPOUND	R_1	R_2	R_3	R_4	R_5	OTHER
DEOXYVERRUCAROL (IIA)	H	H	H	H	H	—
VERRUCAROL (IIB)	H	OH	H	H	H	—
3α-HYDROXYVERRUCAROL (IIC)	OH	OH	H	H	H	—
8α-HYDROXYVERRUCAROL (IID)	H	OH	H	OH	H	—
8β-HYDROXYVERRUCAROL (IIE)	H	OH	OH	H	H	—
16-HYDROXYVERRUCAROL (IIF)	H	OH	H	H	OH	—
9,10-EPOXYVERRUCAROL (IIG)*	H	OH	H	H	H	9,10-EPOXY
8-KETOVERRUCAROL (IIH)	H	OH	—	—	H	8-KETO

*IIG UNDER THE HYDROLYTIC CONDITIONS REARRANGES TO:

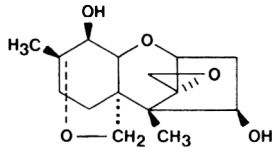


Figure 2. Structures of verrucarols.

determining simple trichothecenes, they have not been particularly useful for the macrocyclic trichothecenes mainly because these compounds lack sufficient volatility to pass through GC columns without appreciable decomposition. Derivatization of these molecules to increase their volatility is usually ineffective. Furthermore, some compounds such as verrucarin J lack easily derivatizable groups such as hydroxyl groups.

Hence, the development of a general GC/MS method for the detection and quantitation of the macrocyclic trichothecenes was undertaken not only because of the obvious challenge, but also they appear to pose a more general threat to public health than is generally recognized (15). It should also be pointed out that some molecules with substituents in the A-ring (see Figure 1) have been found to possess antileukemic properties (6). Bata et al. (16) analyzed the macrocyclic trichothecenes from cultures of 17 isolates of *Stachybotrys atra* grown under laboratory conditions. They separated and measured the major macrocyclic trichothecenes (verrucarin J, roridin E, and satratoxins G and H) by liquid chromatography (LC) and found the total macrocyclic trichothecene content by GC analysis of the verrucarol formed on hydrolysis of the crude extract mixtures. They showed that no verrucarol was present prior to hydrolysis. The macrocyclic trichothecenes, isolated from *Myrothecium* fungal products (17) and *Baccharis* plants (18), on hydrolysis formed both verrucarol and its hydroxylated derivatives. Therefore, we have sought to generate a single, sensitive, and reliable procedure for most of the known macrocyclic trichothecenes via a GC/MS analysis of the hydrolyzed samples suspected to contain a variety of A-ring oxygenated macrocyclic trichothecenes.

Experimental

Apparatus and Reagents

All GC/MS measurements were made using a Hewlett-Packard 5985-B mass spectrometer. Fused silica capillary columns (J&W Associates) were used throughout the investigations. Ultra-pure solvents (Burdick & Jackson), derivatizing agents (Regis Chemicals Co.), micro glassware (Supelco), Sep-Pak disposable cartridges (Waters Associates), and cation exchange resin, DOWEX 50W-X8 (Fisher Chemical) were used.

Table 1. Instrumental (MS) conditions for selected ion monitoring of HFB esters of verrucarols[a]

Group	Compounds (ions) monitored	Scan time, min Start	Run	End
1	Deoxyverrucarol or DOVE (426.2) Scirpentriol (870.3) 8β-Hydroxyverrucarol (870.3)	7.0	1.4	8.4
2	Verrucarol (638.3) 8α-Hydroxyverrucarol (870.3)	8.4	0.4	8.8
3	9,10-Epoxyverrucarol (654.3)[b] 8-Ketoverrucarol (652.3)	8.8	0.9	9.7
4	9,10-Epoxyverrucarol (654.3)[b] 16-Hydroxyverrucarol (870.3)	9.7	1.3	11.0

[a] GC conditions: DB5 fused silica capillary column; spitless injection; column temperature—1 min at 150°C, 10°/min for 10 min, 25°/min to 300°C.
[b] Rearranged product as indicated in Figure 2.

Standard Solutions

Trichothecene standard stock solutions (0.5–2.0 mg/mL) were prepared in methanol in Reacti-vials (Supelco Inc.) fitted with Mininert valves and were stored at 2°C. Dilute standard solutions were prepared using microsyringes and stored similarly. Standard solutions containing all trichothecenes with concentrations of 1 and 10 ng/μL were prepared frequently and used immediately. Internal standards, deoxyverrucarol and 16-hydroxyverrucarol (DOVE and HOVER), were also prepared (10 ng/μL) frequently and the same solutions were used for each series of experiments. Solvent (1 μL) followed by an air column (0.5 μL) were drawn into the microsyringe prior to drawing the derivative solutions for the accurate transfer of standards or samples into the GC column.

Derivatization Procedure

Solutions (1–1000 ng) of standard mixture and internal standard (100 ng) were transferred to a 1.5 mL vial with Teflon-lined screw cap. Solvent was evaporated under nitrogen and the residue was treated with 0.5 mL acetonitrile–toluene (10 + 90) and heptafluorobutyryl imidazole (HFBI, 0.25–0.5 μL). The reaction mixture was shaken well once and kept in a heating block at 110°C for 15 min. After cooling, it was washed twice with 0.4 mL aqueous 5% $NaHCO_3$ solution to remove any acidic impurities formed from excess reagent and water, followed by 0.5 mL water. The solutions containing the same amounts of derivatives were analyzed with or without drying over anhydrous sodium sulfate. Results indicated that drying the derivatives had an adverse effect due to adsorption over the drying agent, especially when present in low picogram quantities, and hence the derivatives were analyzed as such without drying. Traces of water that may be present in the solution did not affect the bonded GC column (DB-5) used during this investigation. The heptafluorobutyryl (HFB) esters were kept in the freezer at −4°C until use. Samples were also derivatized under the same conditions using excess HFBI to ensure the complete derivatization of analytes present in the samples.

Analysis of HFB esters.—One μL of the derivative mixture was injected, using the solvent (toluene—1 μL) wash tech-

SAMPLE
↓ IN METHANOLIC NaOH
↓ AMBIENT TEMPERATURE, OVERNIGHT
CATION EXCHANGE RESIN
↓ METHANOL
↓ EVAPORATION OF ELUATE UNDER N_2
RESIDUE
↓ 3% METHANOL/CH_2Cl_2
SILICA-GEL SEP-PAK CARTRIDGE
↓ 5% METHANOL/CH_2Cl_2
COMBINED ELUATES
↓ EVAPORATION UNDER N_2
RESIDUE
↓ ADDITION OF DEOXYVERRUCAROL (INTERNAL STANDARD)
↓ 10% CH_3CN/TOLUENE, HFBI, 100°, 15 MINUTES
DERIVATIVES
↓ 5%$NaHCO_3$
↓ WATER
ANALYSIS BY GC/NICI-MS

Scheme 1. Hydrolysis and analysis of macrocyclic trichothecenes.

nique, into the gas chromatograph fitted with capillary inlet (Model No. 18835C) and operated in the splitless mode. The injector temperature was maintained at 250°C throughout the investigation. The helium carrier gas pressure in the column was maintained at 10 psi. A fused silica DB-5 (30 m, 0.25 mm, 0.25 μm film thickness) capillary column, directly interfaced to the ionization source, was used throughout this investigation. The GC column was maintained at the initial temperature of 150°C for 1 min, heated at a rate of 10°/min for 10 min, followed by a rate of 25°/min to a final temperature of 300°C, which was maintained for 10 min. The resolved esters were introduced into the source and subjected to chemical ionization at 100°C using methane (0.5–1.0 torr) as the chemical ionization reagent gas. The mass spectrometer was controlled and operated by the data system either to scan from m/z 200 to 960 or to selectively monitor ions as indicated in Table 1. The ion currents or the area of the peaks due to specific ions were measured and used for quantitation of the analytes.

Hydrolysis of macrocyclic trichothecenes.—Known amounts (0.25–5.0 μg) of compound, in 15 mL screw-cap vials fitted with Teflon lining, were treated with 2 mL 1N methanolic sodium hydroxide and left at ambient temperature overnight. The reaction mixture was passed through a chromatographic column (1 × 10 cm) containing the cation exchange resin in methanol. The eluate collected was close to or slightly less than pH 7. The column was further washed with methanol (three 15 mL portions), and the combined eluates were evaporated under nitrogen at 60°C. The residue was immediately dissolved in excess methanol–methylene chloride (10 + 90) (10 mL) and cleaned up using silica gel Sep-Pak cartridges. The cleaned up hydrolysates were converted to their heptafluorobutyryl (HFB) esters and analyzed. Samples containing the macrocyclic trichothecenes were also hydrolyzed and analyzed using this procedure. The cation exchange resin column was reactivated after each use by washing with 3N HCl until the pH of the eluate was strongly acidic. The column was then washed with water (15 mL) and methanol (20 mL).

Discussion

Macrocyclic trichothecenes, the most toxic of the known trichothecenes (4), are either di- or triesters of either unsubstituted or substituted verrucarols (4, 19). The roridins and satratoxins are the diesters of verrucarol, isolated from *Myrothecium* and *Stachybotrys* fungi, respectively (4). The triesters produced by *Myrothecium* fungi are termed as verrucarins (4). These 3 classes of fungi-produced macrocyclic trichothecenes have been implicated in the loss of farm and wild animals and as human health hazards (4–7, 15, 19). The baccharinoids, a group of closely related macrocyclic esters (6) isolated from plants, have antileukemic properties (6). The verrucarins and roridins with substituents in the A-ring, introduced by either synthetic means (20, 21) or microbial transformation (21), also possess the same anticancer properties (6). Hence, it is useful to develop procedures for determining these macrocyclic trichothecenes in order to monitor environmental samples for the presence of these highly biologically active molecules.

During our effort to develop one general procedure of analysis for most of the known macrocyclic trichothecenes, different approaches were pursued. Analysis of underivatized macrocyclic trichothecenes by the GC/MS method was not even attempted since even the less polar simple trichothecenes such as verrucarols (IIA-H) and other simple trichothecenes could not be chromatographed over a GC column

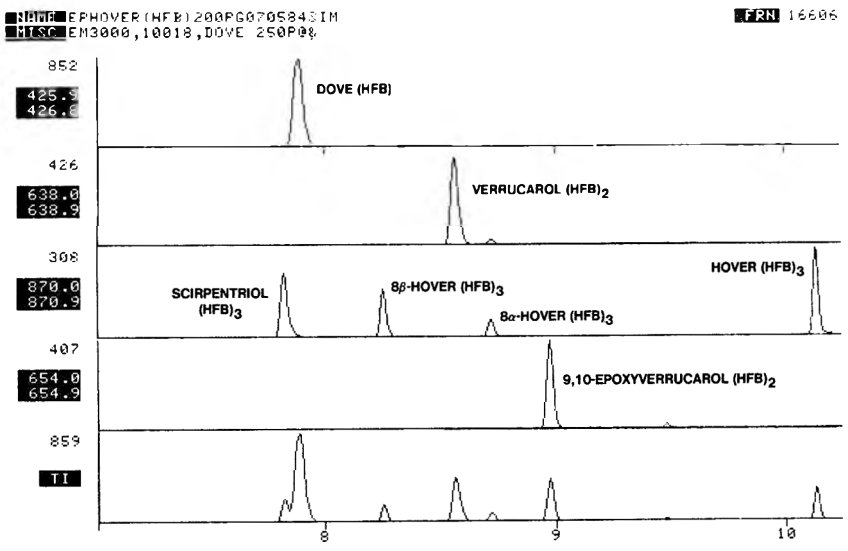


Figure 3. Reconstructed ion chromatograms of verrucarols HFB esters.

Table 2. Calibration data for verrucarol (HFB esters)

Compound	Retention time, min	Ion monitored, m/z	Amounts analyzed, pg	Observed linear range, pg	Linear regression constants: Correlation coefficient	Slope	Intercept
Deoxyverrucarol (HFB)	7.9	426.3	250				
Verrucarol $(HFB)_2$	8.6	638.3	20–1000	20–1000	0.9986	0.5591	−0.0292
Scirpentriol $(HFB)_3$	7.8	870.3	20–1000	20–500	0.9986	0.1996	−0.0067
8β-Hydroxyverrucarol $(HFB)_3$	8.2	870.3	20–1000	20–500	0.9996	0.1477	−0.0018
8α-Hydroxyverrucarol $(HFB)_3$	8.7	870.3	20–1000	50–1000	0.9929	0.0684	−0.0146
16-Hydroxyverrucarol $(HFB)_3$	10.1	870.3	20–1000	50–1000	0.9928	0.3252	−0.0997
9,10-Epoxyverrucarol $(HFB)_2$[a]	9.0	654.3	20–1000	20–1000	0.9912	0.4453	−0.0207
8-Ketoverrucarol	9.3	652.3					

[a] Rearranged product as indicated in Figure 2.

without appreciable decomposition. Hence, the heptafluorobutyrates of roridin A and verrucarin A were chromatographed over a DB-5 capillary column and analyzed by GC/MS technique under chemical ionization conditions monitoring the negative ions. Under these conditions, only the bis-heptafluorobutyrate of verrucarol was detected and the HFB esters of roridin A or verrucarin A were not observed. The results indicated that the GC/MS method is not suited for direct analysis for macrocyclic trichothecenes even after derivatization.

The next logical approach pursued, which turned out to be successful, involved analysis of hydrolysates of the macrocyclic trichothecenes (verrucarols). Roridins, satratoxins, and unsubstituted verrucarins yielded verrucarol on hydrolysis; the baccharinoids and substituted verrucarins formed the corresponding substituted verrucarols. The sample workup protocol, outlined in Scheme 1, was suited for the quantitative determination of the macrocyclic trichothecenes by GC/MS technique.

Prior to hydrolysis of the macrocyclic trichothecenes, the behavior of the various verrucarols under the proposed experimental conditions was studied in detail. Methanolic solution containing known amounts of standards of verrucarol (IIB) and substituted verrucarols (IIC-H) were passed through DOWEX 50W-X8 cation exchange resin under the same conditions intended for the hydrolysates of the macrocyclic trichothecenes. The eluates were evaporated, treated with internal standard, deoxyverrucarol (DOVE) (23), converted to their HFB esters, and analyzed using the SIM conditions as shown in Table 1. During the analysis, characteristic ions of only a few derivatives are monitored at any specific time, thus improving the sensitivity. The intervals selected for monitoring these ions were based on their individual retention times on the GC column. Since none of these derivatives eluted from the GC column prior to 7 min, ion monitoring was started only 7 min after the injection. The derivatives were well resolved under these conditions and the reconstructed ion chromatogram is shown in Figure 3. The stability, linear response ranges during GC/MS analysis, and minimum detectable levels of these derivatives are discussed elsewhere in detail (11). The calibration data for the individual derivatives of standards under these conditions are indicated in Table 2. Such curves are obtained frequently just prior to analysis of the samples. The amount of the verrucarols in the samples was calculated from the observed relative intensities and the corresponding calibration curves obtained, for standards under the identical SIM conditions, just prior to analysis.

The experimentally determined percent recoveries of compounds from the cation exchange resin and silica gel Sep-Pak cartridges (Scheme 1) are shown in Table 3. The verrucarols with the exception of rearranged product of 9,10-epoxyverrucarol were not recovered efficiently from the cation exchange resin column. It is not clear at this point whether the low recoveries were due to the adsorption or degradation of the compounds on the column. The recoveries measured for various verrucarols were in the range of 12–107%. Although most of these values are low, they are highly reproducible as indicated by the low standard deviation values (Table 3) and therefore these data were useful in the quantitation of verrucarols in the samples. Similar recovery results were obtained when the verrucarols were subjected to cleanup procedures over silica gel disposable cartridges. The percent recoveries of verrucarols, when subjected to both these processes, also are listed in Table 3. Despite the low recoveries, these results were encouraging because of the consistency of the measured amounts for these compounds even when only trace (0.25–2.30 μg) amounts were subjected to these multi-step cleanup procedures followed by derivatization and GC/MS analysis. These values could perhaps be improved under carefully controlled experimental conditions and proper quality assurance measures during analysis. These results were encouraging despite the low recoveries considering the consistency in the measured amounts for these compounds even when only trace (0.25–2.30 μg) amounts were subjected to these experimental and cleanup procedures.

Table 3. Percent recoveries of verrucarols

Compound	Amount extracted, μg	Recovery, % (±SD)[a]: 1	2	3
Verrucarol	0.305, 0.610, 1.220	61 ± 4	21 ± 10	19 ± 9
9,10-Epoxyverrucarol[b]	0.250, 0.500, 1.000	122 ± 25	107 ± 8	111 ± 22
3α-Hydroxyverrucarol (scirpentriol)	0.400, 0.800, 1.600	103 ± 5	62 ± 21	57 ± 19
8α-Hydroxyverrucarol	0.575, 1.150, 2.300	62 ± 31	24 ± 1	27 ± 2
8β-Hydroxyverrucarol	0.380, 0.760, 1.520	74 ± 12	31 ± 6	29 ± 11
16-Hydroxyverrucarol	0.330, 0.660, 1.320	39 ± 6	12 ± 4	14 ± 2

[a] 1 = Cartridge treatment only; 2 = resin treatment only; 3 = both treatments.
[b] Rearranged product as shown in Figure 2.

Table 4. Hydrolytic data of macrocyclic trichothecene standards

Macrocyclic trichothecene	Amount hydrolyzed, μg	Hydrolysis product	Amount of hydrolysate, μg		Efficiency, %
			Expected	Detected	
Roridin A	0.862	verrucarol	0.431	0.012	3
	2.155		1.078	0.112	10
	4.310		2.155	0.266	12
Roridin D	0.64	verrucarol	0.321	0.161	50
	1.28		0.642	0.568	89
	2.56		1.285	0.534	42
Roridin H	0.58	verrucarol	0.301	—[a]	—[a]
	1.16		0.603	0.240	40
	2.32		1.205	0.965	80
Satratoxin G	0.25	verrucarol	0.122	0.070	57
	0.50		0.244	0.110	45
	1.50		0.489	0.087	18
Satratoxin H	0.25	verrucarol	0.126	0.055	44
	0.50		0.252	0.040	16
	1.00		0.504	0.303	60
Verrucarin J	1.190	verrucarol	0.648	0.123	19
	2.38		1.285	0.165	13
Verrucarin A	1.110	verrucarol	0.608	0.163	27
	2.220		1.215	0.528	44
3α-Hydroxyverrucarin A	0.540	3α-hydroxyverrucarol	0.294	0.046	16
	1.080		0.588	0.071	12
	1.620		0.882	0.150	17
8α-Hydroxyverrucarin A	0.480	8α-hydroxyverrucarol	0.261	0.126	48
	0.960		0.523	0.095	18
	1.920		1.046	0.131	13
8β-Hydroxyverrucarin A	0.630	8β-hydroxyverrucarol	0.343	0.045	13
	1.260		0.686	0.488	71
	2.52		1.372	0.051	4
16-Hydroxyverrucarin A	0.560	16-hydroxyverrucarol	0.305	0.051	17
	1.120		0.609	0.062	10
	2.240		1.219	0.098	8
9,10-Epoxyverrucarin A	0.590	9,10-epoxyverrucarol	0.321	0.474	148
	1.180		0.642	0.546	85
	2.360		1.285	1.088	86
8-Ketoverrucarin A	0.510	8-ketoverrucarol	0.277	0.067[b]	—
	1.020		0.553	0.513[b]	—
	2.125		1.153	0.590[b]	—

[a] Sample lost during processing.
[b] Relative abundance with respect to internal standard, not the amount of 8-ketoverrucarol.

Known amounts (0.25–4.5 μg) of individual macrocyclic trichothecene standards in triplicates were taken in separate vials and hydrolyzed at ambient temperature using 1N methanolic sodium hydroxide. Each hydrolysate from individual standards was passed through the resin column and silica gel cartridge (Scheme 1). The internal standard, DOVE, was added, analytes were converted to HFB derivatives, and the standards were analyzed by the usual GC/NICIMS procedure

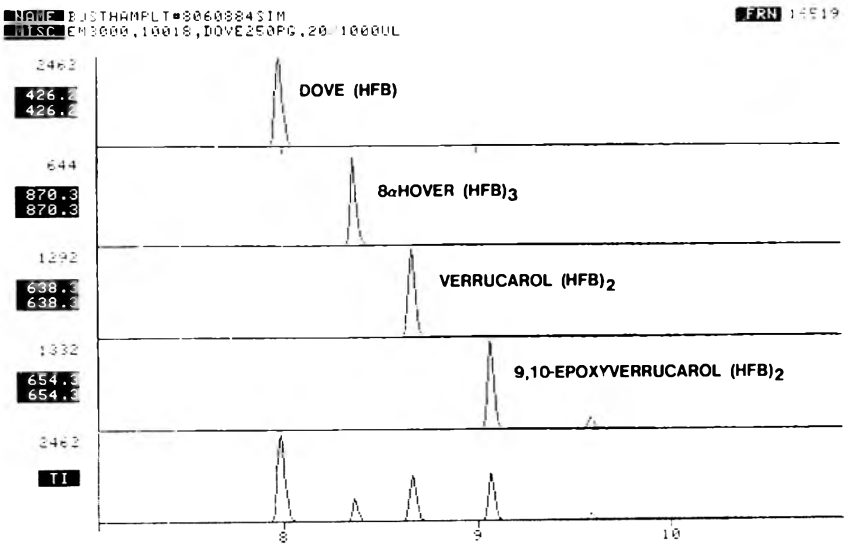


Figure 4. Analysis of hydrolysate of *Baccharis* plant sample.

Table 5. Results from hydrolysis of samples

Sample origin	Fraction of total sample analyzed	Detection ions, m/z	RRT sample[a] (standard)	Compounds identified	Amount in total sample, μg
Myrothecium fermentation broth 1	1/200	638	1.09 (1.09)	verrucarol	147
		870	1.10 (1.10)	8α-hydroxyverrucarol	7
		870	1.28 (1.10)	16-hydroxyverrucarol	0.6
Myrothecium fermentation broth 2	1/200	638	1.09 (1.09)	verrucarol	153
		870	1.10 (1.10)	8α-hydroxyverrucarol	17
Myrothecium fermentation broth 3	1/1000	870	0.99 (0.99)	3α-hydroxyverrucarol	10
		870	1.10 (1.10)	8α-hydroxyverrucarol	205
		870	1.28 (1.28)	16-hydroxyverrucarol	7
		638	1.09 (1.09)	verrucarol	1284
Baccharis plant sample 1	1/1000	870	1.10 (1.10)	8α-hydroxyverrucarol	92
		654	1.14 (1.14)	9,10-epoxyverrucarol	12
Baccharis plant sample 2	1/1000	870	1.04 (1.04)	8β-hydroxyverrucarol	21
		638	1.09 (1.09)	verrucarol	7
		654	1.14 (1.14)	9,10-epoxyverrucarol[b]	17
Stachybotrys fungal sample		638	1.09 (1.09)	verrucarol	—
		870	0.99 (0.99)	3α-hydroxyverrucarol	traces

[a] With respect to DOVE (HFB), retention time, 7.9 min.
[b] Figure 2.

under SIM mode. The amounts of individual verrucarols present in various hydrolysates were measured, and the efficiencies of the hydrolysis and detection of several naturally occurring and the synthetically modified macrocyclic trichothecenes with various verrucarol and ester moieties were determined. The results of these experiments are shown in Table 4. The measured efficiencies, with the exception of the rearranged 9,10-epoxyverrucarin A, were low but consistent. In all instances at least 2 of 3 measured values (Table 4) were consistent, indicating the possibility of the precise quantitation of the macrocyclic trichothecenes in samples. By using determined correction factors for individual compounds, it should be possible not only to detect but also to quantitate nanogram quantities of these macrocyclic trichothecenes with reasonable accuracy. The full scan NICI spectrum of the HFB derivative of the hydrolysate from 8-ketoverrucarin A was consistent with that of the 8-ketoverrucarol. The mass spectrum had strong $(M\text{-}HF)^-$, $(M\text{-}2HF)^-$, and $HFBH^-$ ions along with the M^- ions m/z 672. This is in agreement with the fragmentation of the M^- ions of HFB esters of other verrucarols under the chemical ionization conditions (11). Even though the sample spectrum could not be compared with the mass spectrum of the authentic sample, the identity of the hydrolysate (8-ketoverrucarol) from 8-ketoverrucarin A was quite clear.

Several environmental and fermentation samples, without any prior cleanup, were hydrolyzed and the hydrolysates were processed and analyzed by the usual procedure (Scheme 1). Only an aliquot (1/200–1/5000) of the hydrolysate was derivatized and a fraction (1 μL) of the derivative mixture (500 μL) was sufficient for the GC/MS analysis, due to the high sensitivity of the adapted procedure. The detection and quantitation were done under SIM conditions. The reconstructed ion chromatograms of representative plant and fungal samples are shown in Figures 4 and 5, respectively. The identities of the analytes in total unknown samples were confirmed either by obtaining full scan spectra (Figure 6) or by monitoring 5–6 characteristic ions for individual molecules (Figure 7). The analytical results, obtained for a few of several samples analyzed, are tabulated in Table 5. The samples were from either 50 mL fermentation broth or 50 g plant sample. It should be pointed out again that only a small fraction (1/200–1/5000) of the sample was derivatized and only a minimum amount of the derivative mixture (500 μL) was analyzed. The detected verrucarols were quantitated using calibration curves acquired for individual standards.

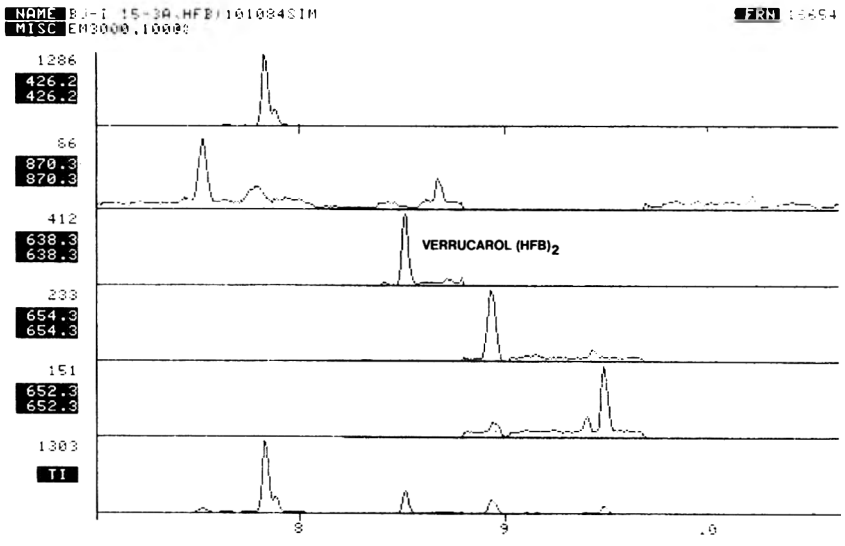


Figure 5. Analysis of hydrolysate of *Stachybotrys* fungal sample.

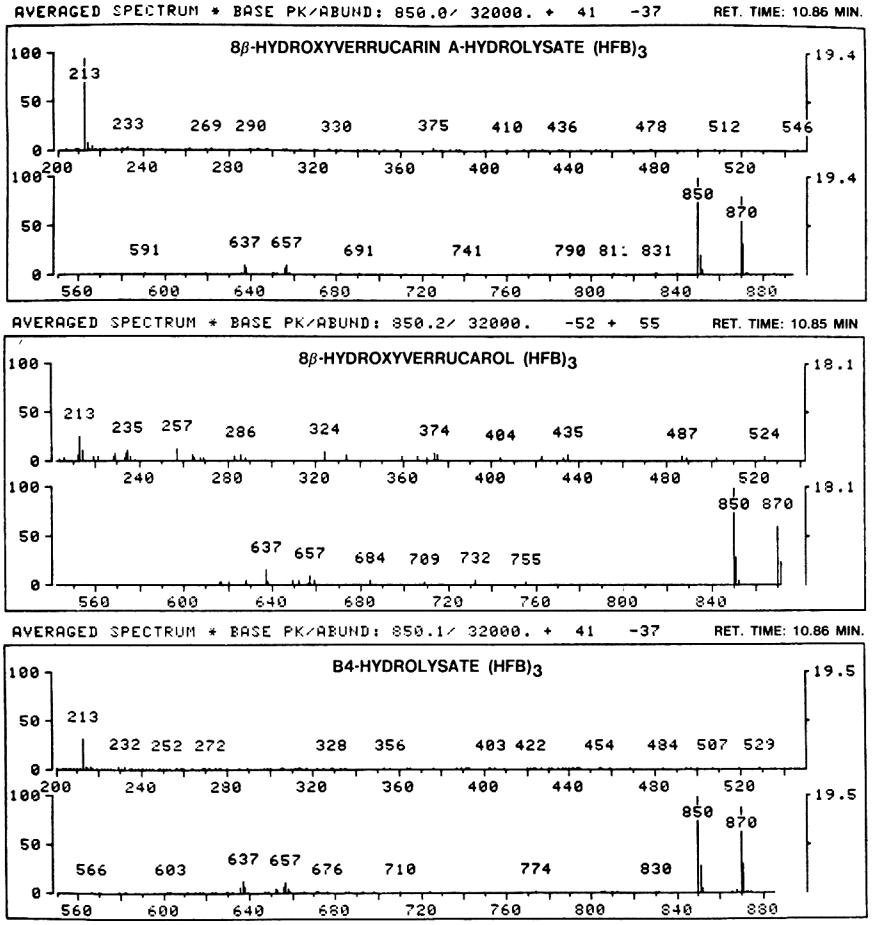


Figure 6. Full scan NICI spectra of 8β-hydroxyverrucarol $(HFB)_3$ and hydrolysates from 8β-hydroxyverrucarin A and B4.

It should be noted that this method detects only the amount of verrucarols in the hydrolysates without identifying the macrocyclic trichothecenes. The verrucarol and the other substituted verrucarols could have originated from roridins, verrucarins, or satratoxins. Even though this is a highly sensitive, reasonably accurate procedure, it provides only the identity of the verrucarol moiety and not that of the ester bridges. This could be overcome in unknown samples by analyzing the original samples under direct chemical ionization conditions, using CH_4 or NH_3, and monitoring either the positive or negative molecular ions to obtain the molecular weight(s) information for the macrocyclic trichothecene(s) present in the sample. This provides only information about the molecular weight of the analytes, not the identity of the macrocyclic trichothecenes. These data in combination with the identity of the hydrolysate could be useful for the identification of the macrocyclic trichothecenes. Unknown samples would be analyzed for the presence of verrucarols prior to hydrolysis as well, in order to confirm the source of verrucarols in the unknowns and also to calculate the amount

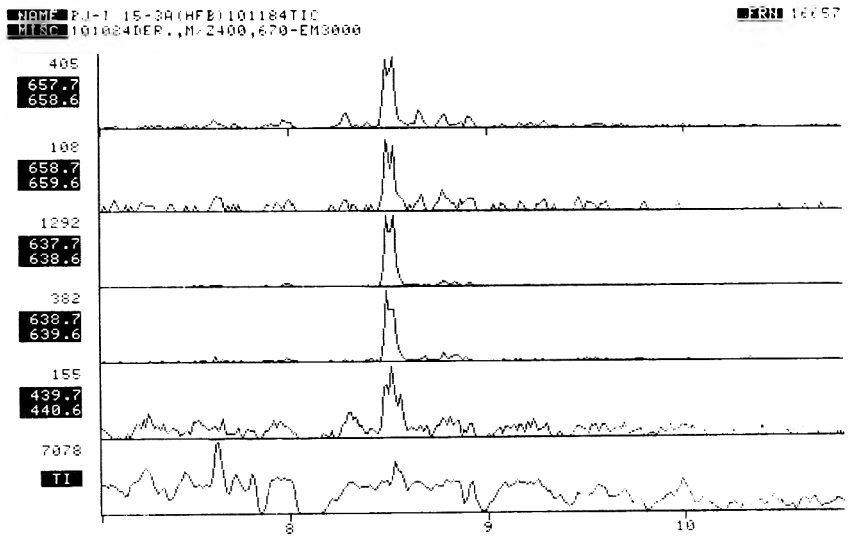


Figure 7. Confirmation of presence of verrucarol in *Stachybotrys* fungal sample by selected ion monitoring.

of verrucarols due to the macrocyclic trichothecenes. Unambiguous identification of components in a mixture should be possible by techniques such as direct chemical ionization-MS/MS, or LC/MS, or Thermospray-MS/MS techniques. Development of such methods for the unambiguous identification and accurate quantitation of macrocyclic trichothecenes by these techniques and evaluation of the developed procedures are under way.

Conclusion

Nanogram (250 ng) quantities of several macrocyclic trichothecenes with different verrucarol moieties and ester bridges were hydrolyzed, and the hydrolysates were unequivocally identified and quantitated with good accuracy using deoxyverrucarol as the internal standard. Accuracy in quantitation could be improved by introducing quality control measures and adequate experimentally determined correction factors in the calculations. The identities of the parent macrocyclic trichothecenes could be determined with their molecular weights determined by DCI-MS technique, if and when required. The quantitation of the marcocyclic trichothecenes in samples was achieved by this procedure. These polar, sensitive molecules, even when present in low ppb levels or lower with adequate concentration of the sample extracts, could be analyzed and quantitated by this method.

Acknowledgments

The authors thank Margeurite Brooks and Dennis Reutter for their interest and valuable suggestions rendered throughout this investigation.

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PLANT TOXINS

Quantitative Determination of Total Glucosinolates in Rapeseed and Meal Digests

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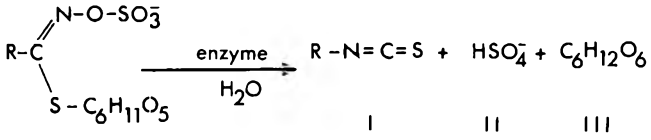
A method has been developed for the quantitative determination of total glucosinolates in enzymic digests of seeds and meals of rapeseed cultivars. The method is based on the determination of the glucose released after myrosinase digestion and subsequent deproteinization, using the glucose oxidase-peroxidase system with 4-aminophenazone as the oxygen acceptor. The myrosinase was prepared from white mustard seeds and partially purified to remove other glucose-liberating enzyme activities. Endogenous enzyme activities in the samples analyzed were also removed by heat treatment before analysis. These conditions allow a rapid and specific estimation of total glucosinolates that is particularly useful in rapeseed breeding programs. The parameters affecting the optimum yield of hydrolytic product (glycose) from the seeds of 2 rapeseed varieties known to contain a high (cv. Target) and a low (cv. Tower) level of glucosinolate contents have been examined. Addition of glucose to the digestion mixtures gave good recoveries (95%) and close agreement between replicates (±5%).

The enzymic breakdown of glucosinolates by myrosinase (thioglucoside glucohydrolase; EC 3.2.3.1) releases various products, namely, glucose, isothiocyanates, and sulfate as:



To determine the levels of glucosinolates in mature seeds and other plant parts of the cruciferous plants, each of these products has been monitored; the methodology has been recently reviewed by McGregor et al. (1). Isothiocyanates (I) have been estimated by gas chromatography (2–5), by ultraviolet absorption after conversion to thioureas (5, 6), by other chemical methods (7–9), and more recently by liquid chromatography (10,11). Other methods, rarely used, estimate the released sulfate (II) gravimetrically (12) or the released glucose (III) enzymically (13–15). Assays of the anomeric form of D-glucose based on the specificity of D-glucose oxidase (β-D-glucose: oxygen oxide reductase, (EC 1.1.3.4) for β-D-glucose have involved β-D-glucose oxidase–peroxidase system as:

$$\beta\text{-D-Glucose} + \underset{\text{oxidase}}{\text{glucose}} + O_2 \rightarrow \underset{\text{acid}}{\text{gluconic}} + H_2O_2$$

$$H_2O_2 + \text{peroxidase} + \underset{\text{(colorless)}}{O_2 \text{ acceptor}} \rightarrow H_2O + \underset{\text{(colored)}}{\text{oxidized acceptor}}$$

A major disadvantage with the glucose oxidase–peroxidase-based reagents used until recently was that they included oxygen acceptors (13, 14) which, without exception, are known or suspected carcinogens. However, Trinder (16) recently introduced a new, noncarcinogenic oxygen acceptor, 4-aminophenazone, into the glucose oxidase–peroxidase system for the assay of glucose in blood.

Bjorkman (17) applied the glucose oxidase–peroxidase system to the analysis of enriched glucosinolate fractions but concluded that the procedure was not suitable for crude solutions that contained colored or other interfering substances. Although VanEtten et al. (14) partially overcame this problem by removing interfering substances by charcoal treatment, the charcoal also adsorbed the liberated glucose to the extent of 5–15%. In addition, the procedure used a crude enzyme preparation for the liberation of glucose and did not remove the protein prior to color development. We report a method which employs a noncarcinogenic oxygen acceptor, a deproteinizing agent to remove protein and interfering substances, and a partially purified enzyme preparation free of interfering activities. This permits the use of commercially available glucose oxidase and peroxidase to measure glucosinolate levels by a simple, reliable, and economical procedure that is particularly useful in the analysis of rapeseed breeding lines.

Experimental

Reagents

(a) *Samples.*—Rapeseed was obtained from a collection of germplasm used as breeding lines in the New South Wales Department of Agriculture. Seeds were high quality, viable, and free from extraneous material. White mustard seeds were procured locally.

(b) *Solvents.*—AR grade except where specified.

(c) *Enzymes and substrates.*—Glucose oxidase Type V from *Aspergillus niger* and peroxidase Type II from horseradish (Sigma Chemical Co.); sinigrin (allylglucosinolate hydrate) (Aldrich Chemical Co.); *p*-nitrophenyl glycosides (Sigma Chemical Co.); 4-aminophenazone (Sigma Chemical Co.).

(d) *Protein precipitating reagent.*—20 g sodium tungstate ($NaWO_4 \cdot 2H_2O$),20 g disodium hydrogen phosphate (anhydrous), and 18 g NaCl were dissolved in 700 mL water, and pH was adjusted to 3.0 by adding 125 mL 2N HCl. Then 2.0 g phenol was added and volume was adjusted to 1 L with water.

(e) *Glucose oxidase–peroxidase reagent.*—Solution of 10 mg peroxidase plus 200 mg 4-aminophenazone in 10 mL water was added to 580 mL (1%, w/v) anhydrous disodium hydrogen phosphate containing 10 mL glucose oxidase solution. Sodium azide, 600 mg, was added as preservative and the solution was stored at 4°C. Solution will keep about 1 month.

(f) *Working glucose standard.*—2 g desiccated glucose (dried under vacuum over phosphorus pentoxide) was dissolved in 1 L saturated benzoic acid to a final concentration of 200 mg/100 mL.

(g) *Tris-HCl buffer.*—1.6 g trizma base and 13.7 g trizma HCl were dissolved in 950 mL water, pH was adjusted to 7.0, and volume was adjusted to 1 L.

Received May 20, 1985. Accepted May 15, 1986.

Table 1. Substrate specificity of myrosinase from *Sinapis alba* seeds

Substrate	Enzyme	Activity[a]
Sinigrin	myrosinase	100
p-NO_2-phenyl-α-D Glc p	α-D-glucosidase	<0.5
p-NO_2-phenyl-β-D Glc p	β-D-glucosidase	<0.5
p-NO_2-phenyl-α-D Glc p	α-D-glucosidase	<0.5
p-NO_2-phenyl-β-D Glc p	β-D-glucosidase	<0.5
Sucrose	invertase	<1.0

[a] Glucose (μg) released in the same period that the enzyme releases 100 μg from sinigrin.

(h) *Sodium acetate buffer.*—16.5 g anhydrous sodium acetate and 11.5 mL concentrated acetic acid were added to 950 mL water, pH was adjusted to 4.9, and volume was adjusted to 1 L.

(i) *Myrosinase solution.*—10 mg lyophilized myrosinase was dissolved in 1.0 mL sodium acetate or Tris-HCl buffer.

Procedure

Preparation, partial purification, and kinetic properties of myrosinase (Thioglucoside glucohydrolase; EC 3.2.3.1).—One hundred g frozen seeds of white mustard was finely milled in a coffee grinder, the flour was extracted with 500 mL cold sodium acetate buffer in an Ultra-Turrax blender, and the suspension was stored 2 h at 4°C. The extract was centrifuged 15 min at 14 000 × *g* and 4°C, and 70% saturated ammonium sulfate solution was added to the clear supernate which was stored 2 h at 4°C. The precipiate was recovered by 15 min centrifugation at 14 000 × *g* and 4°C and redissolved in a minimum of water. This solution was dialyzed against 2 changes of water for 24 h at 4°C and centrifuged again 15 min at 14 000 × *g* and 4°C to remove residual protein. Ice cold acetone was added to the clear supernate to 70% concentration and the supernate was stored overnight at 4°C. The precipitate, recovered by centrifugation 15 min at 14 000 × *g* and 4°C, was dissolved in minimum volume (150 mL) of cold water and freeze-dried. In this form, the enzyme was very stable, and this preparation was used in kinetic studies and for determination of total glucosinolate levels in rapeseed and meal samples.

Each time a new preparation was made, the activity of the enzyme as optimized using sinigrin as substrate. To a 1.0 mL solution of 5mM sinigrin in either Tris–HCl or sodium acetate buffer, 0.05 mL enzyme solution was added and the solution was incubated 30 min at 37°C. The reaction was terminated and released glucose was estimated by the addition of glucose oxidase–peroxidase reagent to determine activity as described in a later section. Optimum pH activity was checked using 5mM sinigrin in water as substrate in either Tris-HCl or sodium acetate buffer in pH range 4.5–9.0. Effect of ascorbic acid on hydrolysis of 5mM sinigrin was determined using concentrations of 1–25mM. The apparent Michaelis constant for the enzyme was determined at pH 7.0 and 37°C with sinigrin concentrations of 1–10mM.

To determine substrate specificity, 0.1 mL myrosinase solution was incubated with 0.1 mL M/40 nitrophenyl glycosides at 30°C for 15 min. The reaction was stopped by adding 2.8 mL 2% sodium carbonate. The nitrophenyl released was measured at 420 nm.

Preparation of rapeseed and meal homogenates.—Five mL boiling water was added to 30–50 mg sample of whole seeds in conical test tube, and the tube was incubated 5 min at 100°C. Tubes were cooled, and water was removed with a Pasteur pipet. Seeds were homogenized in a micro mortar and pestle with 2.0 mL Tris–HCl buffer, and transferred to a 10 mL graduated conical test tube. The mortar and pestle were washed twice with 1.0 mL buffer each time and washings were added to the tube.

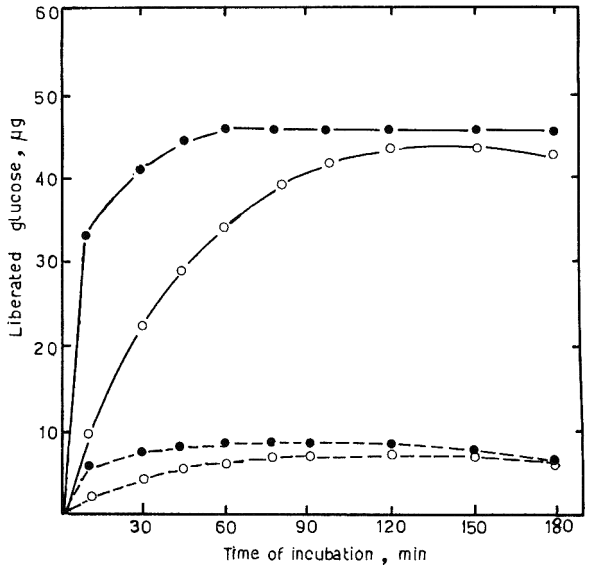


Figure 1. Liberation of D-glucose on glucosinolate hydrolysis when homogenates from 250 mg rapeseed (cv. Tower - - - and cv. Target ———) were incubated with 0.5 mL enzyme solution (10 mg/mL) in 10 mL 0.10M Tris-HCl buffer, pH 7.0 (●——●) and 0.10M acetate buffer, pH 4.9 (○——○) at 37°C. 1.0 mL aliquots were drawn at 10, 30, 45, 60, 75, 90, 120, 150, and 180 min for glucosinolate assay.

Meal samples, 20–50 mg, were directly boiled in 2 mL Tris-HCl buffer for 5 min, transferred to micro mortar and pestle, homogenized, and transferred to tubes as above. Final volume of homogenates, both seeds and meal, was adjusted to 5.0 mL with Tris–HCl buffer.

Hydrolysis of glucosinolates and estimation of glucose.—To seed or meal homogenates, 0.05 mL of 10 mg/mL enzyme solution was added with thorough mixing. Tubes were incubated 2 h in 37°C water bath, with occasional shaking. Reaction was stopped by adding 5.1 mL protein precipitating reagent and tubes were gently shaken and centrifuged 5 min at 5000 × *g* or allowed to stand 30 min.

A 0.1–1.0 mL aliquot of clear solution was transferred to a 10 mL test tube and adjusted to 1.0 mL with half-diluted protein precipitating reagent (v/v in water). Three mL glucose oxidase–peroxidase reagent was added and all tubes were immediately incubated 15 min at 37°C. Absorbance was read immediately at 515 nm in a UV spectrophotometer. Substrate blanks were run concurrently to allow correction for free endogenous glucose.

Calculations.—Results were expressed as μmoles total glucosinolates per g sample as follows:

Total glucosinolates/g = (μg glucose per aliquot)/(mol. wt glucose) × (total ext vol./aliquot vol.) × (1/g sample)

where weight of glucose in aliquot was read from the calibration curve prepared by incubating known amounts of glucose, 0–100 μg, with color reagent under the same conditions.

Results

To investigate and optimize the incubation conditions to give complete hydrolysis of glucosinolates and optimum yield of the reaction products, a method was required which stopped the reaction immediately without causing losses of the hydrolysis product (glucose) and which did not interfere with its determination. The glucose oxidase–peroxidase system

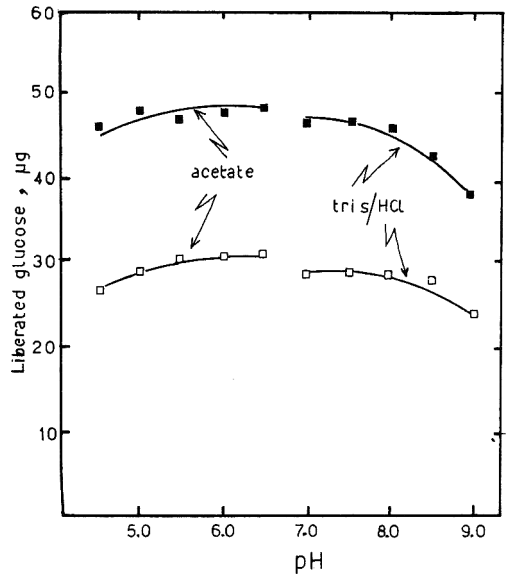


Figure 2. pH dependence of D-glucose formation on glucosinolate hydrolysis when homogenate from 25 mg rapeseed Target (■——■) and 5mM sinigrin (□——□) were incubated 60 min with 0.05 mL enzyme (10 mg/mL) in acetate (0.1M) and Tris-HCl (0.1M) buffer of varying pH at 37°C.

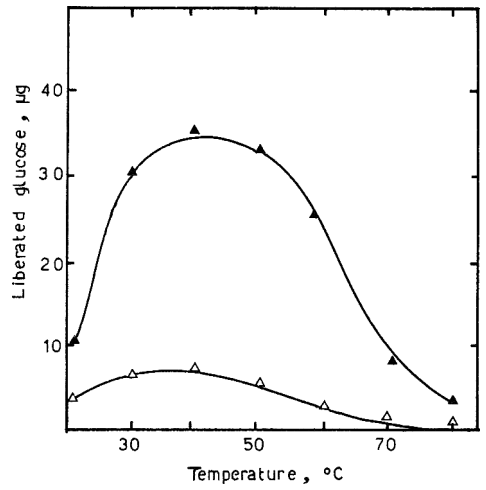


Figure 3. Effect of temperature on formation of D-glucose on glucosinolate hydrolysis when homogenates from 25 mg rapeseed Target (▲——▲) and Tower (△——△) were incubated 60 min with 0.05 mL enzyme (10 mg/mL) in Tris-HCl buffer, (pH 7.0, 0.1M) at varying temperatures.

includes 4-aminophenazone as the oxygen acceptor and phenol–tungstate reagent to stop the reaction in the rapeseed and meal digests and also to act as a protein precipitant. Phenol–tungstate is also essential for color development because it contains the phenol and forms part of the buffer system.

Incubation conditions were standardized periodically using seeds of 2 varieties known to contain high (cv. Target) and low (cv. Tower) levels of glucosinolates. Glucose in the digests after the reaction was stopped was determined as described in the preceding section. Samples were analyzed in duplicate and the incubation conditions are described in the caption to each figure.

The standardized conditions were used in the analysis of total glucosinolates in seed and meal samples of rapeseed breeding lines.

Specificity of the enzyme.—Rapeseed and mustard seeds contain galactosyl-sucrose oligosaccharides and amyloids (18, 19), both of which contain glucose. It is thus essential that the myrosinase used is devoid of enzyme activities which will liberate glucose from other polymers. The partially purified preparation of myrosinase used in these investigations was checked for various glucose-liberating activities (Table 1), and myrosinase was the only glucose-liberating activity present.

Time course of liberation of D-glucose on glucosinolate hydrolysis.—The results summarized in Figure 1 show that the reaction is rapid at pH 7.0 and is complete in less than 60 min. At pH 4.9, the reaction proceeds slowly and reaches a plateau after 120 min. The amount of glucose liberated at pH 4.9 after 2 h is less than that released at pH 7.0. Addition of more enzyme (0.1 mL) to the digests and prolonged incubation did not increase glucose values.

Effect of pH on formation of D-glucose.—The effect of pH on the amount of D-glucose released under standard assay conditions was studied by preparing rapeseed homogenates in buffers of varying pH and incubating with aliquots (0.05 mL) of enzyme solution. Above pH 6.5, Tris–HCl buffer was used. Sinigrin (5mM) was prepared in freshly distilled water. A broad pH optimum, in the range 5.5–8.0 (Figure 2), similar to those found by Appelqvist and Josefsson (8) was confirmed.

Effect of temperature on release of D-glucose.—To establish the optimum incubation temperature, we studied the temperature dependence of the reaction. Rapeseed homogenates prepared in Tris–HCl buffer were incubated with enzyme at varying temperatures for 60 min. Maximum liberation of glucose occurred between 30 and 40°C (Figure 3). At temperatures above 60°C, values were much lower.

Effect of myrosinase concentration on liberation of D-glucose.—The effect of enzyme concentration on the liberation of glucose from rapeseed homogenates (25 mg seed sample) is shown in Figure 4. An enzyme concentration of 0.25 mg/25 mg seed gave maximum release of glucose and was used in routine analyses. Similar results were obtained when sinigrin was used as a substrate. Ascorbic acid has been reported by some investigators (20) to stimulate myrosinase activity. However, it was not included in the current assay procedure because of its inhibitory effects on peroxidase in the glucose oxidase–peroxidase system (21). A 22% inhibition in the color reaction was observed at 1mM ascorbic acid and it gave complete inhibition at 2.5mM.

Precision and Accuracy

The precision and accuracy of the test were improved by denaturing endogenous enzyme activities in the material being analyzed. This was achieved by incubating the seeds in a boiling water bath. To check the degree of hydrolysis, the reaction was carried out according to the procedure described earlier. The reaction was stopped by heating the tubes in a boiling water bath for 5 min. After cooling, the mixture was transferred to a dialysis bag and dialyzed 24 h at 4°C against 2 changes of water. The combined dialysates were concentrated by rotary evaporation (below 40°C) to 5.0 mL. A portion of the solution was assayed for glucose. Another portion, after equilibration against Tris–HCl buffer, was incubated (37°C, 60 min) with enzyme and assayed for glucose. Also, the mixture from the dialysis bag was transferred to a test tube and suspended in Tris–buffer, enzyme solution was added, and the mixture was reincubated at 37°C for 60 min. No further liberation of glucose was detected either in the dialysate or the mixture. Therefore, under the conditions used, hydrolysis of the glucosinolates in the seed and meal homogenates was complete. In a third experiment, a series of parallel tests were run with and without added glucose

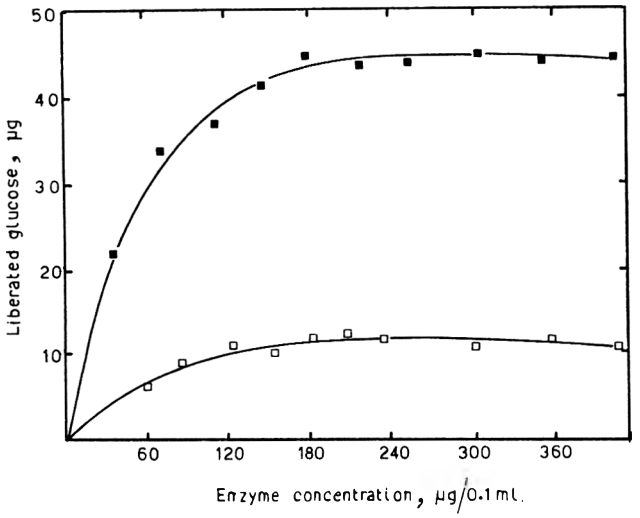


Figure 4. Effect of myrosinase concentration on formation of D-glucose from homogenates of rapeseed Target (■——■) and Tower (□——□) incubated 60 min in Tris-HCl buffer (pH 7.0, 0.1M) at 37°C.

(0.6 mg glucose/5 mL homogenates from 25 mg seeds of cv. Target) in the reaction mixture. Recovery of added glucose was 95% under the standard assay conditions. When triplicate analyses on seed and meals of a number of cultivars were performed (Table 2), agreement between the replicates (±5%) was good. The overall means for seeds and meals were 48.66 and 53.7 µmoles/g sample, respectively, and the average standard deviation per sample was 2.65 µmoles/g sample. The standard errors did not differ between seeds and meals, but did vary with the level of the response. However, analysis on less than 10 mg sample is not recommended because sampling error then becomes significant.

Several major and minor (about 50) glucosinolates differing only in the R-group of the compound have been identified in the plants of the Cruciferae family (22). Their hydrolysis yields glucose and sulfate quantitatively when catalyzed by myrosinase (23) with different products (e.g., isothiocyanates) arising from the aglycone moiety. Therefore, the estimation of glucosinolates by measuring myrosinase-released glucose has several advantages over gas chromatography (GC) and liquid chromatography (LC) as well as methods based on sulfate determination, because the first is simple, precise, and highly specific. GC methods, although used for many years, have several disadvantages. Methods of Youngs and Wetter (2) do not measure all individual glucosinolates (isothiocyanates). Glucosinolates, after conversion to their trimethylsilyl (TMS) derivatives and subsequent analysis by GC (3, 4) produce peaks of major and a number of minor but unmeasurable compounds; this causes a cumulative error in true value of total glucosinolates. Furthermore, GC methods are unsuitable for glucosinolates with hydrophobic side chains (24) and some aromatic glucosinolates (25, 26). It is well known that some glucosinolates produce products other than isothiocyanates by treatment with myrosinase (24, 25), and a loss in products due to TMS derivatization cannot be overlooked. The use of LC methodology as an analytical tool for quantitative analysis of glucosinolates is still in its infancy. A lack of suitable methods for the preparation of glucosinolate fractions from plant constituents prior to LC analysis may pose problems similar to GC analysis. Moreover, high costs involved in establishing and running such procedures often render them unfeasible as routine analytical tools for screening large populations of early generation breeding lines.

Although the potential of glucose oxidase–peroxidase reaction mechanism as a determinant of total glucosinolates in *Brassica* species has been investigated by some workers (13, 14), its application as a routine analytical method in a breeding program has often been ignored. This could be attributed to a number of problems encountered in the estimation of glucose after the seed and meal samples are treated with enzyme. Previous investigators experienced difficulties in removing the protein or other interfering substances prior to color reaction (14, 17).

Table 2. Glucosinolate content of seeds and meals of various rapeseed cultivars[a]

Cultivar	Seed,[b] µmoles/g		Meal,[c] µmoles/g	
	Mean	SD	Mean	SD
Forto	81.77	3.26	93.30	2.78
Victor	68.87	1.70	73.87	4.55
Gulle	60.47	2.50	61.73	2.76
Brink	46.13	2.31	48.20	1.83
Midas	83.93	3.45	92.23	6.58
Major	76.20	3.70	84.13	0.99
Duro	36.20	1.11	38.90	3.92
Yellow Sarson	91.88	4.16	102.23	3.40
Target	76.27	1.81	81.27	2.23
Tower	19.03	3.23	21.10	1.61
Alter	17.93	3.25	18.97	3.15
Regent	15.97	1.20	17.83	1.42
RR-9	14.90	1.39	16.37	1.90
RR-15	14.27	1.82	16.27	0.93
Torch	47.47	4.19	58.23	3.85
Wesroona	16.62	1.18	19.27	1.82
Wesbell	59.30	3.67	65.10	2.36

[a] Each value represents a mean of 3 replicates.
[b] Corrected for total oil content (determined by Soxhlet extraction method) and moisture on 7.5% basis.
[c] Corrected for moisture on 7.5% basis.

The principal requirements of analytical methods designed to screen early and late generation breeding materials are speed, simplicity, specificity, accuracy, and, above all, cost-effectiveness. Although Craig and Morgan (15) modified the glucose oxidase–peroxidase system for analyzing glucosinolates by using expensive automated equipment, this still did not eliminate the use of charcoal prior to glucose estimation. The method described in this paper enables one person to manually analyze 20 samples in duplicate per day. Whole seed samples can be analyzed without defatting, thus offering an advantage over the method of Craig and Morgan (15).

In the present method, the problems experienced by the previous investigators have also been overcome. A large proportion of the colored substance is removed by boiling the seed samples before hydrolysis and the residual protein is precipitated from solution prior to glucose estimation. Provided the hydrolysis conditions are accurately established, this method provides a highly specific, accurate, and precise measurement of total glucosinolates in seeds and meals of rapeseed. However, to apply this method to other *Brassica* species, a check on the hydrolysis conditions, inactivation of endogenous glucose-releasing enzymes, and a determination of free glucose in the samples are advised.

The method can be applied to very small samples or single seeds with appropriate modifications. It provides an efficient, accurate, inexpensive, and specific means of screening and selecting plants low in glucosinolate content and thus, is directly applicable to breeding programs.

Acknowledgments

The authors thank S. Heffer and S. Scott for technical assistance and J. Sorgini for assistance in the preparation of

this manuscript. Thanks are also due to B. V. McCleary, Senior Research Scientist, Biological and Chemical Research Institute, Rydalmere, for valuable suggestions and criticism.

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SYMPOSIUM ON CHROMATOGRAPHY OF AMINO ACIDS

99th Annual International Meeting of AOAC, October 1985

(*Editor's note.*—Four of the 8 presentations made during the Symposium on Chromatography of Amino Acids at the 99th AOAC Annual International Meeting, October 27–31, 1985, are published in this issue of the *Journal of the AOAC*. The remaining 4 will appear in the next issue.)

"Chromatography of Amino Acids: Sample Preparation, Gas Chromatography (Packed Column and Capillary), Liquid Chromatography, and Ion-Exchange" was the subject of a symposium held at the 99th Annual International Meeting of AOAC, October 27–31, 1985, in Washington, DC, and cochaired by Charles W. Gehrke and Robert W. Zumwalt. The objective of this symposium was to present the current state of the art of amino acid analysis, from techniques for protein hydrolysis to the latest developments in the chromatographic analysis of amino acids, including gas chromatography (GC), ion exchange chromatography (IEC), and liquid chromatography (LC). The invited speakers were chosen on the basis of their recognized contributions to the field of amino acid analysis.

Robert W. Zumwalt presented "Hydrolysate Preparation for Amino Acid Determination," which reviewed an extensive study of variations in the classical 6N HCl, 110°C, 24 h hydrolysis method. Variations included the use of glass tubes with Teflon-lined screw caps as the hydrolysis vessel; high-temperature, short-time hydrolysis; performic acid oxidation of cystine and methionine; multiple hydrolysis times at 145°C; and interlaboratory preparation of hydrolysates. For a wide, diverse sample set, the screw-cap tubes gave results in good agreement with those for sealed ampoules, and hydrolysis at 145°C, 4 h gave results in agreement with 110°C, 24 h. The intra- and interlaboratory variability of chromatographic or analytical steps involved in the measurement of amino acids, whether by ion-exchange or gas chromatography, is less than the variability produced by the sample hydrolysis preparation step performed by different laboratories. Thus, hydrolysate preparation is a crucial step and requires the most attention to solve the problems of variation in amino acid analyses.

Samuel L. MacKenzie led in the development of the *N*-heptafluorobutyryl isobutyl derivatives of amino acids for gas chromatographic analysis. In his presentation entitled "Gas Chromatographic Analysis of Amino Acids as the *N*-Heptafluorobutyryl Isobutyl Esters," MacKenzie noted that these derivatives are well resolved by gas chromatography and form the basis of a convenient, rapid assay. A complete protein amino acid profile can be completed in less than 20 min with a packed column and more rapidly with capillary columns. He recommended use of capillary columns for analyses of physiological samples and use of a nitrogen-specific detector for identification and measurement of nonprotein amino acids in physiological samples.

Charles W. Gehrke presented "Analysis of Amino Acids as the *N*-Trifluoroacetyl *n*-Butyl Esters," which described derivatization conditions and dual column analyses of a variety of sample types. He presented the comparison of gas chromatographic and ion-exchange chromatographic analyses of the same hydrolysates of 9 diverse sample types. GC–IEC percent differences in values from the same hydrolysates averaged only 3.6%, and a similar comparison of results from hydrolysates prepared by different laboratories showed a mean difference of 6.3%. Gehrke described the development and application of GC analysis for determination of amino acids in a broad range of biological substances, including sample purification, derivatization, and chromatography. Floyd Kaiser described his experiences in the routine use of the method for more than 15 years. These studies demonstrate that GC and IEC results from a range of sample types are essentially identical when applied to the same hydrolysate.

Robert M. Schisla described the importance of the accurate measurement of the sulfur amino acids cystine and methionine in poultry rations and methionine supplementation of rations for optimum performance. Charles Gehrke then presented "Quantitative Analysis of Cystine, Methionine, Lysine, and Nine Other Amino Acids by a Single Oxidation–4 Hour Hydrolysis Method." In that presentation describing a joint research effort with Monsanto, Gehrke and his coworkers established that lysine and 9 other amino acids can be determined accurately in proteinaceous materials which have undergone performic acid oxidation. This analytical methodology addresses the need of the private sector for a rapid method for cystine, methionine, lysine, and other amino acids in poultry feed and feed ingredients.

Ernst Bayer presented "Capillary GC Analysis of Amino Acids by Enantiomeric Labeling." Bayer has been a pioneer in the gas chromatographic analysis of amino acids, and his development of optically active liquid phases for capillary GC separation of amino acid enantiomers has resulted in an "enantiomeric labeling" technique in which the D-isomers serve as internal standards for quantitative analysis of the naturally occurring L-isomers. In addition, he has developed an automated system for derivatization and GC analysis of amino acids which is applicable to a broad range of sample types.

Brian Bidlingmeyer presented "New Technique for Rapid Quantitative Analysis of Amino Acids Using Precolumn Derivatization." He has extensively studied liquid chromatographic techniques for amino acid analysis and described the use of phenylisothiocyanate for precolumn derivatization of amino acids. He reported that the method is rapid, efficient, sensitive, and specific for the analysis of primary and secondary amino acids.

Joseph DiBussolo discussed "Liquid Chromatography and Protein Analysis–Solutions to Analytical Problems." In his presentation on the chromatographic separations of proteins and peptides, he described the importance of the fidelity of gradient formation due to the strong dependence of protein retention on mobile phase strength and reported that poor mass yields and peak distortions due to nonspecific protein adsorption to various LC components can often be alleviated by simple washing procedures. He noted that detection limits can be extended by on-line buffer purification and by the reduction of refractive index effects that occur during gradient elution.

Raymond B. Ashworth described "Ion-Exchange Separation of Amino Acids with Post-Column OPA Detection," which focused on the essential amino acid analysis of mechanically separated red meat and poultry products. The method he has adapted to LC analysis utilizes post-column hypochlorite oxidation, OPA derivatization, and fluorescence detection.

Ashworth described gradient formation and the detection of hydroxyproline and proline, and he noted that sample preparation is a critical part of the method. A defatting procedure is used for removal of fat and other nonprotein nitrogenous substances, and a hydrolysis procedure is used to protect tryptophan. However, corrosion damage to the equipment by halide buffers has brought about a search for alternative methodology.

James R. Benson presented "High Performance Amino Acid Analysis Using Polymeric Columns," noting that post-column derivatization of free amino acids after separation on polymeric ion-exchange columns is an accurate and reliable analytical method. He described highly efficient columns that permit analyses of protein hydrolysates in less than one-half hour and noted that the high chemical stabilities of the materials provide long column lifetimes.

"Capillary GC Analysis of Amino Acids with Bonded Phase Fused Silica Columns" was presented by Robert W. Zumwalt as the closing paper of the symposium. He described the separation and analysis of the protein amino acids as the *N*-trifluoroacetyl *n*-butyl esters and the *N*-heptafluorobutyryl isobutyl esters. His paper described the application of capillary GC analysis for quantitative measurement of amino acids by using bonded phase columns which are commercially available and in wide use for a broad range of applications. Analyses of samples ranging from pure proteins to mixed feeds illustrated that, with simply the purchase of a fused silica capillary column and the appropriate reagents, any laboratory with the ability to perform capillary GC analysis can perform quantitative measurements of amino acids.

Following the final presentation, an open discussion was held in which the assembled speakers responded to a range of questions from the audience. Thus, the symposium provided an excellent forum for exchange and dissemination of the most recent information on amino acid analysis to interested scientists from a range of disciplines.

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Acid Hydrolysis of Proteins for Chromatographic Analysis of Amino Acids

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The conditions used to hydrolyze proteins are vital in determining amino acid compositions because they necessarily represent a compromise aimed at yielding the best estimate of amino acid composition. Variations in ease of peptide bond cleavage, differences in amino acid stabilities, and matrix effects from nonproteinaceous components all militate against a single set of hydrolysis conditions that quantitatively hydrolyze every peptide bond and concurrently cause no destruction of any amino acid. This presentation summarizes and reviews an extensive study which evaluated a number of variations in the techniques and procedures of the classical 6N HCl, 110°C, 24 h hydrolysis of protein. The objectives of the recent investigation were: (*1*) to compare hydrolysis at 145°C, 4 h with 110°C, 24 h for proteins in a wide range of different sample matrixes; (*2*) to compare protein hydrolysis at 110°C, 24 h conducted in sealed glass ampoules after vacuum removal of air with hydrolysis in glass tubes with Teflon-lined screw caps after removal of air by vacuum, nitrogen purge, and sonication; (*3*) to evaluate a performic acid oxidation procedure before hydrolysis for the analysis of cystine and methionine in the different sample matrixes; (*4*) to evaluate multiple hydrolysis times at 145°C; (*5*) to evaluate the variation of interlaboratory hydrolysates prepared at 145°C, 4 h in 2 different laboratories on the amino acid analysis of an array of protein-containing matrixes. The major sources of inaccuracy and lack of precision arising from the application of ion-exchange or gas chromatography, both of which provide excellent accuracy and precision, are prechromatographic sample handling and the method used for hydrolysis of the protein sample itself. Hydrolysate preparation is the area that requires the most attention to solve problems of variability of amino acid analyses.

Dramatic advances in chromatography have increased sensitivity, resolution, versatility, and reliability for measurement of amino acids in biological samples during the past 30 years. However, for amino acid analysis of proteins, one remaining major limiting parameter determines both the precision and accuracy with which the amino acid composition can be determined and the speed and cost with which those data can be obtained. That limiting parameter is the reliable preparation of the sample hydrolysate for the chromatographic method.

Over the years, hydrochloric acid has emerged as the most widely used reagent for hydrolysis of proteinaceous substances for determination of amino acid composition. The conditions used to hydrolyze proteins with HCl are vital in determining amino acid compositions because they necessarily represent a compromise aimed at yielding the best estimate of amino acid composition. Variations in ease of peptide bond cleavage, differences in amino acid stabilities, and matrix effects from nonproteinaceous components all militate against a single set of hydrolysis conditions that quantitatively hydrolyze every peptide bond and concurrently cause no destruction of any amino acid (1–7).

The pioneering work of S. Moore and W. H. Stein in development of ion-exchange methodology for analysis of amino acid mixtures made possible initial studies to define and develop procedures for protein hydrolysis.

Accepted June 9, 1986.
Presented at the Symposium on Chromatography of Amino Acids, 99th AOAC Annual International Meeting, Oct. 27–31, 1985, Washington, DC.
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Correspondingly, gas chromatographic techniques have played a central role in amino acid analysis, offering advantages in resolution, sensitivity, speed, and cost. The most widely used approach has been derivatization of the amino acids to their *N*-acylated amino acid esters before gas chromatographic analysis. In 1959, Youngs (8) successfully analyzed 6 amino acids as their *N*-acetyl-*n*-butyl esters, and in 1962, Zomzely et al. (9) resolved a mixture of 19 *N*-trifluoroacetyl *n*-butyl esters. In 1965, Lamkin and Gehrke (10) reported the first quantitative studies on the analysis of amino acids by gas chromatography and selected the *N*-TFA *n*-butyl derivatives.

Gehrke, Zumwalt, Kuo, and coworkers went on to publish some 30 articles on amino acid analysis by gas chromatography, ranging from studies of derivatization reactions (11–16) to high sensitivity analyses of the returned lunar samples (17, 18). The 2 major derivatives that have evolved are the *N*-TFA *n*-butyl esters described above and the *N*-heptafluorobutyryl isobutyl esters developed by MacKenzie (19). Extensive reviews (19–23) of the earlier development of gas chromatography have been published.

Reverse phase liquid chromatography (LC) for amino acid analysis has been investigated, based on derivatization with dansyl chloride (24–27), *o*-phthalaldehyde (28–31), phenylisothiocyanate (32), and other derivatizing agents. Commercial systems are now being offered for reverse phase LC amino acid analysis.

Review articles on the hydrochloric acid hydrolysis of proteins to amino acids (1–7) discuss factors such as the concentration and purity of the acid, hydrolysis time and temperature, the presence of carbohydrates in the matrix, effects of aldehydes and metal impurities, and the kinetics of protein hydrolysis and amino acid destruction, and they describe numerous strategies for improving the yield of refractory and labile amino acids.

In 1985, Gehrke et al. (33) published the results of an extensive study which evaluated a number of variations in the techniques and procedures of the classical 6N HCl, 110°C, 24 h hydrolysis of protein, which this presentation will summarize and review. Those variations were: (*1*) the use of glass tubes with Teflon-lined screw caps as the hydrolysis vessel; (*2*) high-temperature, short-time hydrolysis; (*3*) performic acid oxidation of cystine and methionine; (*4*) multiple hydrolysis times at 145°C; and (*5*) interlaboratory preparation of hydrolysates.

Evaluation of Classical Hydrolysis of Protein

Our experience and that of others (34) has been that sample handling and hydrolysis procedures produce greater errors in determining the amino acid composition of proteinaceous materials than do the chromatographic methods of analysis. Collaborative trial results, ably reviewed by Williams (34), served to focus attention on the paramount issue of proper sample preparation and hydrolysis in obtaining accurate amino acid composition data, whether for pure proteins or samples containing substantial amounts of other material.

The most ambitious and successful collaborative study, one conducted within the European Economic Community, was reported by Andersen et al. (7), who used a streamlined hydrolysis procedure they had developed earlier. Fifteen amino acids are typically measured by this procedure, with losses of only tyrosine and histidine occurring during the oxidation step. In the collaborative study, participants hydrolyzed 5 common feeds: barley meal, fish meal, grass meal, skim-milk powder, and soybean meal; each participant analyzed these hydrolysates by ion-exchange chromatography along with a common standard amino acid solution and a test solution. Also, the participants returned portions of the hydrolysates for analysis by the coordinating laboratory in Copenhagen. For the accepted data from the participants' analyses, the mean coefficients of variation (CVs) for repeatability ranged from 2.47 to 5.09%, with the mean CVs for reproducibility ranging from 5.09 to 10.56%. Analyses of the participants' hydrolysates by the single coordinating laboratory yielded within-laboratory mean CVs ranging from 1.86 to 3.23%, and between-laboratory mean CVs ranging from 2.45 to 5.24%.

Table 1. Protein sample set representative of different matrixes

Sample No.	Sample Type	Description
1	soybean meal	soybean oil meal from AAFCO Check Feed Series, 7921
2	poultry feed	feed sample from AAFCO Check Feed Series, 7931
3	fish meal	commercial protein feed ingredient
4	wheat	hard red winter variety
5	Pruteen	commercial single cell protein feed ingredient, ICI, Ltd
6	NBS Orchard Leaves	National Bureau of Standards, SRM 1751
7	NBS Bovine Liver	National Bureau of Standards, SRM 1577
8	egg white	freeze-dried egg white product
9	ribonuclease	ribonuclease A, Type I-A, Sigma Chemical Co.

The hydrolysis method used in the collaborative study was developed by Mason, Bech-Andersen, and Rudemo (35) in Denmark after extensive studies on the preparation of hydrolysates for amino acid analysis of feed constituents. They studied the effect of the acid/nitrogen ratio on amino acid recoveries from feed proteins hydrolyzed under reflux with 6N HCl (36), the influence of hydrolysis time on amino acid recovery (37), and the effect of phenol and formic acid on the amino acid recovery from oxidized feed proteins (38). Their data showed that the yield of amino acids was consistent over a 40-fold change in the acid/nitrogen ratio, that 110°C, 24 h hydrolysis under reflux was justified for most amino acids, and that the isoleucine, valine, and serine values should be increased by 6% to correct for incomplete recoveries of these amino acids. They also concluded that the inclusion of phenol in the oxidation and hydrolysis mixtures protected labile amino acids such as phenylalanine, histidine, and arginine, and markedly reduced the losses of tyrosine, and that the presence of formic acid in the final hydrolysate had no serious detrimental effects on chromatography and recovery. Further, they developed more streamlined procedures for preparation of hydrolysates which compared well with conventional procedures (39).

In the recent investigation by Gehrke et al. (33), we examined 5 variations of classical protein hydrolysis. First, hydrolysis at 145°C, 4 h was compared with 110°C, 24 h for proteins in a wide range of different sample matrixes. Second, protein hydrolysis at 110°C, 24 h conducted in sealed glass ampoules after vacuum removal of air was compared with hydrolysis in glass tubes with Teflon-lined screw caps at the same temperature after removal of air by vacuum, nitrogen purge, and sonication. Third, a performic acid oxidation procedure before hydrolysis was evaluated for the analysis of cystine and methionine in the different sample matrixes. Fourth, multiple hydrolysis times at 145°C were evaluated.

Fifth, the variation of interlaboratory hydrolysates prepared at 145°C, 4 h in 2 different laboratories was evaluated on the amino acid analysis of an array of protein-containing matrixes.

The sample set for these studies was selected to contain a broad spectrum of sample matrix types. Included were plant, animal, and microbiological protein sources, American Association of Feed Control Officials Check Feed Samples, National Bureau of Standards reference materials, and a pure protein, ribonuclease. The description of the sample set is given in Table 1.

One important objective was to compare the classical acid hydrolysis technique (2) with 2 other hydrolysis procedures for determination of amino acid content of an array of sample matrixes. The samples were selected on the basis of diverse protein content and matrix variability.

Results and Discussion

The classical method of hydrolysis at 110°C, 24 h in a sealed glass ampoule was compared with hydrolysis at 145°C, 4 h and with a method using glass tubes with Teflon-lined screw caps at 110°C, 24 h.

The sums of 15 amino acids as determined by all 3 hydrolysis treatments showed generally excellent agreement. However, for the 2 animal protein samples, fish meal and bovine liver, the sum of amino acids by hydrolysis in screw-cap tubes was about 3.5% lower than the sum by the other 2 treatments.

We compared the precision of the amino acid results for hydrolysis with the reference sealed glass ampoule and the screw-cap tube at 110°C, 24 h: the precision is appreciably better for the screw-cap tube (RSD, % = 1.40) than for the sealed glass ampoule (RSD, % = 2.17).

Recoveries of 15 amino acids after hydrolysis with a screw-cap tube, 110°C, 24 h compared with those after hydrolysis with a sealed glass ampoule, 110°C, 24 h or 145°C, 4 h showed that recoveries following hydrolysis when a screw-cap tube was used were excellent for most samples. Again, however, the total recovery by the screw-cap tube procedure was low for the 2 animal proteins mentioned above, fish meal and bovine liver, by 3.8 and 3.6%, respectively. Also, ribonuclease recovery was 4.1% lower following hydrolysis in a sealed glass ampoule at 145°C, 4 h than at 110°C, 24 h, the classical method.

Furthermore, results from our experiments demonstrated the necessity of prehydrolysis oxidation with performic acid to obtain quantitative data for cystine and methionine. The cystine recovery without oxidation was acceptable only for the pure protein, ribonuclease. The average recovery for all samples was 55.5%. In the case of methionine, recoveries without oxidation are acceptable for several of the samples. Methionine recoveries without oxidation of the samples were excellent for those samples high in protein and low in carbohydrate: fish meal, Pruteen, NBS Bovine Liver, egg white, and ribonuclease, averaging from 94.4 to 104.6% of the oxidized values. However, for other samples, methionine recoveries were low. Because performic acid oxidation is required for the quantitation of cystine, methionine should also be determined as methionine sulfone in the oxidized sample.

Effects of Hydrolysis Time and Temperature on Threonine, Serine, Valine, and Isoleucine

The effects of temperature and time of hydrolysis on the yield of 4 of the amino acids affected by these parameters were studied. Threonine and serine are susceptible to destruction during hydrolysis, while the peptide bonds in which valine and isoleucine are involved are difficult to hydrolyze. In addition, hydrolysis condition comparisons were made for 3 amino acids which are both stable to acid hydrolysis and easily hydrolyzable: aspartic acid, alanine, and leucine. Hydrolysis at 145°C, 4 h was compared with hydrolysis at 110°C, 24 h for these 3 amino acids; no appreciable differences were found between the 2 hydrolysis conditions.

The recoveries of threonine and serine after hydrolysis at 145°C, 4 h were compared with those at 110°C, 24 h, and as expected, the higher temperature resulted in a greater destruction of these amino acids, with serine generally lost to a greater degree than threonine. The yields of valine and isoleucine, amino acids with peptide bonds that are difficult to hydrolyze, were greater at 145°C, 4 h than at 110°C, 24 h.

Multiple Hydrolysis Times and Extrapolation

Hirs et al. (1) suggested that 2 hydrolysis times, 20 and 70 h at 110°C, were required for maximum recovery of amino acids; Roach and Gehrke (3) in their studies using hydrolysis at 145°C also showed that multiple hydrolysis times were necessary to achieve maximum recoveries for threonine, serine, valine, and isoleucine. In our recent studies (33), we selected 3 samples having different matrixes to evaluate the effect of multiple hydrolysis times on the amino acid results obtained. We found that 2 hydrolysis times can be used to determine accurately the zero intercept. The hydrolysis times of 24, 32, 48, and 72 h gave the best fit of the points to a line (lowest root mean square error), and the zero-intercept value for 2 hydrolysis times, 24 and 72 h, was in good agreement with the 4 hydrolysis times zero-intercept value: 4.28 and 4.30 for threonine, and 6.85 and 6.90 for serine. It is also important that one of the 2 hydrolysis times should be 24 h at 110°C, because that is the reference time for the other amino acids.

A sealed ampoule hydrolysis time study was then repeated for threonine and serine on egg white at the 145°C hydrolysis temperature. The results showed that hydrolysis times of 4 and 16 h yield extrapolated zero-intercept values near the maximum value. Similar hydrolysis time studies at 145°C were performed with 2 other sample matrixes: soybean meal and wheat. Again, 4 times, 4, 6, 8, and 16 h, gave the best linear fit, and 2 hydrolysis times (4 and 16 h) agreed closely with the 4-time calculated value. Average recoveries for threonine and serine in 3 matrixes, using a single 4 h hydrolysis time, were 95.4 and 90.8%, respectively, of the values derived from multiple-time hydrolysis.

Maximum recoveries of valine and isoleucine from the sealed ampoule hydrolysis time studies at 145°C for egg white, soybean meal, and wheat samples were obtained at a hydrolysis time of 16 h. The percent recoveries were computed from the 4 h hydrolysis values relative to the 16 h values for these 2 amino acids. When the results on egg white from the sealed ampoule, 110°C, 72 h hydrolysis were compared with the sealed ampoule, 145°C, 16 h hydrolysis, the valine and isoleucine recoveries were 94.5 and 96.8%, respectively; thus, hydrolysis for valine and isoleucine under the 145°C, 4 h conditions gives higher values and is simpler and faster.

Recovery of the essential amino acids in all 3 samples, egg white, soybean meal, and wheat, was compared for the 2-hydrolysis time, 145°C treatment and the sum of the essential amino acids by conventional 110°C, 24 h hydrolysis. The average recovery from the 145°C, 4 h treatment was 102.3% of that obtained from the latter hydrolysis.

Samples of purified egg white and wheat flour were selected to determine the effect of variations in hydrolysis time at 145°C. It was observed that the variation in results for any

of the amino acids on either sample was small over the 3–5 h hydrolysis interval; however, a 4 h hydrolysis was considered optimum for both sample types.

Interlaboratory Comparison of Hydrolysates Prepared at 145°C, 4 Hours

The variations in an interlaboratory comparison of hydrolysates were evaluated by providing a set of 9 samples to Analytical BioChemistry Laboratories (ABC), a commercial laboratory, to prepare the hydrolysates according to the procedure described by Gehrke et al. (33). The hydrolysates were then returned to the University of Missouri Experiment Station Chemical Laboratories (ESCL) laboratory and analyzed with a corresponding set of hydrolysates prepared by ESCL and analyzed on the Beckman 121 M analyzer. The average relative percent differences between the ESCL- and ABC-prepared hydrolysates for the different matrixes of all 15 amino acids ranged from 2.56% for egg white to 5.17% for NBS Orchard Leaves.

The sum of the 15 amino acids found in the hydrolysates prepared at the 2 laboratories showed that amino acids of the 9 sample types differed by absolute percentages of only 0.21% for egg white and up to 2.86% for wheat.

For an overview of which amino acids tend to be more subject to variations in interlaboratory hydrolysis methods, the percent differences between the individual amino acid values for the 9 samples were averaged. The data show that for a wide range of sample matrixes, hydrolysates prepared by 2 different laboratories can be expected to yield amino acid values differing by less than 5%. The amino acids generally regarded as the most sensitive of the 15 amino acids to interlaboratory variation in hydrolysis methods, serine and isoleucine, gave the 2 highest average differences, 9.97 and 5.08%, respectively.

Precision of Analysis

We investigated the precision of ion-exchange chromatography of amino acids as a function of the concentration of amino acids in the sample solution. As expected, the precision of analysis is improved as the concentration of the amino acids in the sample solution increases and the amount injected to the analyzer is greater.

In general, for analysis of protein hydrolysates, 10 μg of total amino acids is injected, corresponding to 125 nanomoles/mL of each amino acid in the sample and giving an RSD of about 0.5%.

Conclusions

The demand for quantitative amino acid composition analysis continues to increase in both industry and public research institutions, partly in response to economic and nutritional value considerations of agricultural products. Amino acid analysis finds application in a multitude of fields of research such as biochemistry, biomedical science, molecular biology, environmental science, biogeochemistry, chemical evolution, and the now fast-developing field of biotechnology. It is important to note that the area devoted to assessment of the nutritional value of foods and feedstuffs ranks high on the scale of importance. The nutritive value of protein and proteinaceous foods and products depends on their amino acid composition and essential amino acid balance.

The literature is replete with approaches to amino acid analysis. The 2 major techniques available, ion-exchange and gas chromatography, both provide excellent precision and accuracy. Therefore, it has become increasingly evident that the major inaccuracies and lack of precision which arise from the application of these methods result from the prechromatographic sample handling and the method used for hydrolysis of the protein itself. Whether by ion-exchange or gas chromatography, the intra- and interlaboratory variations in steps involved in the measurement of the amino acids produce much less varibility than does the hydrolysate preparation step done by different laboratories.

This report focuses on an in-depth study of various hydrolysis procedures and emphasizes the quantitative measurements of amino acids susceptible to decomposition and those that are refractory to hydrolysis. This study also provides a feeling for some of the difficulties that arise and how, in part, they might be overcome. Further, we investigated the effects of the hydrolysis method, time, and temperature, and biological matrixes on the precision and accuracy of amino acid analysis using practical, routine methods. The findings in these studies, along with those of the European Economic Community study, can form the basis for development of AOAC collaborative trials to establish the interlaboratory performance of sample hydrolysis and amino acid analysis. Such trials would be of great benefit both to laboratories currently performing amino acid analyses and to those who wish to do so in the future.

The classical 6N HCl hydrolysis procedure is typically performed at 110°C, 24 h in glass ampoules that are flame-sealed after removal of air. We evaluated glass tubes with Teflon-lined screw caps as a more convenient alternative hydrolysis vessel, using 6N HCl hydrolysis conditions at 110°C, 24 h, and also 145°C, 4 h.

From this study on the hydrolysis and amino acid analysis of 9 diverse sample types, we found that the use of glass tubes with Teflon-lined screw caps and hydrolysis at 145°C, 4 h results in amino acid recoveries equivalent to those obtained by use of sealed ampoules; the Teflon-lined screw-cap tube technique is easy, fast, and reproducible.

Hydrolysis at 145°C, 4 h yields results comparable to the classical sealed ampoule 110°C, 24 h hydrolysis, and thus is a more rapid means of preparing proteinaceous samples for analysis on a routine basis. For analysis of cystine, prehydrolysis oxidation is required, and the analysis of methionine as methionine sulfone is preferable and performed on the same oxidized sample. If research demands highly accurate values for serine, threonine, valine, and isoleucine, preparation of 2 hydrolysates for different times in Teflon-lined screw-cap tubes at 145°C with extrapolation to infinite time for valine and isoleucine and to zero time for serine and threonine is recommended. The interlaboratory hydrolysis study demonstrated that the 145°C, 4 h hydrolysis procedure is reproducible, with the data in close agreement, between laboratories for a wide range of sample types. Furthermore, we found that glass tubes with Teflon-lined screw caps as the hydrolysis vessel compared favorably with sealed glass ampoules in terms of amino acid recovery, reproducibility of hydrolysate preparation, and ease of handling. We thus conclude that 145°C, 4 h hydrolysis in glass tubes with Teflon-lined screw caps, with careful exclusion of air, is a practical, rapid, and precise method for hydrolysis of proteins for amino acid analysis by ion-exchange, reverse phase, or gas chromatography.

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Gas Chromatographic Analysis of Amino Acids as the *N*-Heptafluorobutyryl Isobutyl Esters

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The *N*-heptafluorobutyryl isobutyl derivatives of proteic amino acids are well resolved by gas chromatography and form the basis of a convenient, rapid assay. The derivatives are prepared by acid-catalyzed esterification at 120°C for 20 min in 3N HCl-isobutanol followed by acylation with heptafluorobutyric anhydride at 150°C for 10 min. The reaction sequence is performed without any transfers or extractions and thus is compatible with microscale analysis. A complete proteic amino acid profile can be completed in less than 20 min by using a packed column or less than 10 min by using a capillary column in combination with an elevated oven temperature program rate. Physiological sample matrixes, which frequently contain a complex mixture of components, and thus require maximum resolution, can be assayed in less than 1 h using a program rate of 4°C/min. A capillary column is recommended for this application. Capillary column chromatography, in combination with a nitrogen-specific detector, is useful for identifying and assaying nonproteic amino acids in physiological sample matrixes. Frequently, a prior cleanup of the sample can be avoided.

Amino acids are inherently nonvolatile and can be analyzed by gas chromatography (GC) only after appropriate volatile derivatives are formed. Although a wide variety of derivatives has been studied (1–3), the methods now most commonly used are all based on the formation of *N*(*O*,*S*)-acylated amino acid alkyl esters. Originating in the work of Youngs (4), who separated the *N*-acetyl *n*-butyl esters of 6 amino acids, this derivatization strategy was put on a firm foundation by the studies of Gehrke and his colleagues (5, 6). Specifically, this group was responsible for developing procedures for the quantitative, reproducible formation of *N*-trifluoroacetyl (TFA) amino acid *n*-butyl esters and for their chromatographic separation. Those procedures were subsequently applied, largely without modification, to other amino acid alkyl esters and other perfluoroacyl derivatives.

Since resolution of *N*-TFA *n*-butyl esters of the proteic amino acids requires the use of 2 chromatographic columns, the obvious advantages of a single-column system stimulated studies of other derivatives. The first such separation of *N*-acyl alkyl esters of the proteic amino acids was achieved by Moss et al. (7) using the *N*(*O*,*S*)-heptafluorobutyryl (HFB) *n*-propyl esters. Subsequently, the *N*-HFB isoamyl esters (8) and *N*-acetyl *n*-propyl esters (9) were also resolved.

These derivatives offered excellent resolution of the proteic amino acids in the equimolar amounts present in a standard

Accepted June 9, 1986.
Presented at the Symposium on Chromatography of Amino Acids, 99th AOAC Annual International Meeting, Oct. 27–31, 1985, Washington, DC.

amino acid mixture but were not necessarily adequate for real samples containing widely differing amounts of certain amino acids. For example, a small amount of methionine would be difficult to assay, as the *N*-HFB or *N*-acetyl *n*-propyl ester, in the presence of a large amount of aspartic acid. Similarly, the *N*-HFB isoamyl esters would not be the derivatives of choice for assaying small amounts of lysine and tyrosine in the presence of a relative abundance of glutamic acid.

The discovery of these restrictions, while they were analyzing seed protein hydrolysates, led MacKenzie and Tenaschuk (10) to develop a single-column separation based on the *N*-HFB isobutyl esters. By using these derivatives, they completely resolved methionine, aspartic acid, and glutamic acid from the other proteic amino acids, and the procedure was therefore suitable for the analysis of a variety of physiological samples. Subsequent developments included detailed studies of the optimum conditions for esterification (11) and acylation (12) and application of the procedure to the assaying of specific amino acids (13–15), free amino acids in physiological samples (16), and protein hydrolysates (17, 18).

Others have also contributed significantly to development of GC analysis of amino acids as the *N*-HFB isobutyl ester derivatives. For example, Siezen and Mague (19) studied the derivatization and chromatography of 50 biologically interesting amino acids. The resolution of *N*-HFB isobutyl amino acids on a capillary column was first reported by Pearce (20). Later applications involving the use of capillary columns have focused on biological systems, specifically clinical (21, 22) and environmental (23, 24). The advantages of sensitivity and specificity arising from the use of electron capture (24, 25) and nitrogen specific detectors (21) have been described. The "on-column" injection mode has been noted to be preferable for quantitative capillary amino acid analysis (26).

The reader is directed to more comprehensive sources for further details on the development, implementation, and applications of this procedure (3, 27, 28).

Sample Preparation

Proper sample preparation is crucial to a successful analysis; compounds which can interfere with the derivatization or the chromatography must be removed. Purified proteins are usually derivatized directly after hydrolysis, but more complex molecules such as lipoproteins and glycoproteins require the removal of the lipid and carbohydrate moieties, respectively. Free fatty acids definitely interfere with the chromatography and should be removed by standard extraction procedures (23). When free amino acids are assayed, protein must first be removed, because, even under the essentially anhydrous conditions used for esterification, partial hydrolysis of protein can occur. Deproteinization is most commonly achieved by standard procedures such as precipitation with picric acid, trichloroacetic acid, or sulfosalicylic acid. This and other aspects of the overall process of assaying amino acids by GC have been critically reviewed (29).

Further purification of the protein-free sample is usually achieved by cation-exchange chromatography. The procedures have been sufficiently well documented that it is sufficient, in the present context, to draw the reader's attention to some which have been specifically applied to sample preparation for assaying amino acids by GC. For example, Gehrke and his associates developed precise procedures for cleanup of biological samples such as plasma and urine (30), and procedures have been described for dealing with small volumes of plasma or urine (6, 9, 21, 22, 31).

Ion-exchange procedures for the cleanup of biological and geological samples ranging from soil to marine sediments to beer, wine, and similar products have been reviewed elsewhere (3).

Regardless of the specific procedure being used, factors common to the preparation and operation of ion-exchange columns are important. Cancalon and Klingman (32) described rules which should be followed to minimize extraneous GC peaks. Control experiments to assess the recovery of each amino acid should be performed, and the correction factors thereby obtained should be applied to the analysis (9).

Under certain circumstances, excellent results can be obtained without elaborate sample cleanup. Derivatization, in combination with analysis by GC, in effect, also contributes to sample cleanup. Compounds which are nonvolatile, which do not derivatize, or which produce derivatives having a mass too large to permit elution under normal chromatographic conditions are not observed.

Sample Derivatization

Apparatus

Microscale derivatization is most easily performed with 1 mL or 300 μL Reacti-Vials (Pierce Chemical Co., Rockford, IL 61105) or their equivalent. The important features of these vials are a thick glass wall, a reservoir which is conically shaped at the bottom, and Teflon-faced seals. Smaller vials (100 μL) are also available.

The vials may be heated in an oven (20), an oil bath, or a metal heating block (9, 11, 19). When a heating block is used, better heat transfer is obtained by adding oil or bath wax to the sample well (19). Immersion of a vial in the oil only to the level of the fluid inside the vial produces a vertical temperature gradient which causes the solvent to reflux.

The cleanliness of glassware requires special attention. Cancalon and Klingman (32) washed all glassware with hot 1N NaOH, followed by thorough rinsing with distilled water, washing with hot 50% HNO_3, rinsing with double distilled water, and finally drying at 100°C. Alternatively, heating to 450–500°C for 2–6 h will remove contaminating organic material (19). Rinsing with a variety of organic solvents may be adequate for some samples.

Efficient derivatization requires the complete removal of reagents after each reaction, but particularly after esterification. Complete removal has been effected by freeze drying (8, 33), but evaporation by using a stream of dry nitrogen is more common (9, 11, 19–21, 34, 35). Difficulties in reproducible derivatization using nitrogen evaporation (33) do not seem to be general, but, depending on economic factors and the sample load, freeze drying (33) or rotary evaporation (36) may be advantageous under certain circumstances.

Reagents

Before dealing with the preparation of specific reagents, some important general considerations must be mentioned. Reagents and solvents are used in several hundredfold excess and thus even trace impurities may ultimately be in excess in relation to the sample. This applies particularly to high-boiling impurities, which may not evaporate along with the reagent and so could remain to interfere in subsequent reactions. In the author's experience, reagent impurities are the single factor most responsible for problems. Therefore, *analytical grade* reagents and solvents are recommended. This recommendation includes antioxidants, water scavengers, and reagents such as acetyl chloride which may be used to gen-

erate HCl in situ. Furthermore, all solvents must be anhydrous, should be redistilled from an all-glass apparatus, and should be stored over a molecular sieve in tightly sealed containers. Every batch of fresh reagent should be periodically checked in a control derivatization to ensure its purity. Nitrogen or any other gas used to evaporate reagents should be filtered to trap hydrocarbons and water.

Acidified isobutanol is most conveniently prepared by adding acetyl chloride (270 μL/mL produces 3M) to isobutanol. Direct addition of HCl gas is less convenient (11), but, in principle, preferable because the resultant system contains fewer components.

Extraneous chromatographic peaks have been attributed to the presence of carbonyl compounds (32, 34, 37). If long-term storage is contemplated, prudence would suggest storage at −20°C. Good analytical practice requires that the main stock be appropriately stored and that daily or weekly supplies be transferred to a smaller container.

Felker (38) observed peroxide accumulation in ethyl acetate, and a periodic test is recommended.

Reagent grade heptafluorobutyric anhydride (HFBA) is definitely not satisfactory (12, 38). Perhaps it is not entirely coincidence that results obtained in this laboratory have not been duplicated elsewhere when reagent grade HFBA has been used (20, 21, 33).

Reagent purity and preparation is described in greater detail elsewhere (3, 28), and a thorough study by Rash et al. (39) is recommended for information on other potential sources of contamination.

Esterification

Drying of an aqueous amino acid solution before esterification has been effected by freeze-drying (8, 33) but evaporation using a stream of dry nitrogen is more common (11, 19, 34). Methylene chloride may be added to remove the last traces of water azeotropically (19). However, this step has been considered unnecessary after evaporation in a nitrogen stream at 70°C (34). Adding methylene chloride to the esterification reaction has been reported to improve the yield of the hydroxy amino acids and ornithine and tryptophan when forming the *n*-butyl esters (32) but to have no effect on the isobutyl esters (11).

Some amino acid hydrochlorides have limited solubility in the higher alcohols. Unsuccessful attempts to form the isobutyl ester of cystine at a concentration of 2 mg/200 μL of reagent may simply reflect the limited solubility of cystine in the alcohol (40). In this laboratory, cystine is typically derivatized at a concentration some 50- to 100-fold less.

Amino acid solubilization has been promoted by sonicating the reaction mixture. Sonication times as long as 30 min have been studied (32) but, under different conditions, 15–20 s (34) or 10 min (19) may be sufficient. Instead of sonication, the writer has routinely removed the vial from the heating block after about 5 min and agitated the contents using a Vortex Genie Mixer (Scientific Industries, New York, NY).

Water scavengers such as dimethoxypropane have been claimed to promote ester formation (9), but other authors have not found them necessary (11, 19) and they are not generally used.

Acylation

The conditions used for formation of the *N*(*O*,*S*)-HFB derivatives of amino acid alkyl esters vary considerably. March (41) obtained reproducible formation of *N*-HFB *n*-propyl methionine and histidine only by adding an antioxidant, 2,6-di-*tert*-butyl-*p*-cresol (BHT) to the reaction mixture. Similarly, Pearce (20) and Felker (38) obtained better yields of *N*-HFB isobutyl histidine and tryptophan, respectively, when adding BHT, but others have observed no significant effect on the yield of di-HFB isobutyl tryptophan (12) and HFB methionine (42). Siezen and Mague (19) also obtained good yields of HFB methionine, histidine, and tryptophan without the use of antioxidants. The contradictory results reported by different authors cannot readily be resolved. The positive results obtained with antioxidants may be related to the presence of oxidizing contaminants in reagent grade HFBA. If this hypothesis is correct, it is preferable to purify the reagent than to add yet another compound which must itself be purified. No compound should be added unless its use has been unequivocally established to be necessary; therefore, the addition of antioxidants is not recommended. Furthermore, the fewer components there are in the reagent system, the easier it is to control experimental variables and to identify the source of problems.

Special factors must be considered in respect to the acylation of arginine, tryptophan, and histidine. Although the HFB isobutyl amino acids have been formed at 110°C (20, 21), the preponderance of evidence in the literature indicates that a temperature of 150°C is required to obtain a quantitative yield of the arginine derivative (12).

The yield of di-HFB tryptophan depends on temperature (12) and the ratio of tryptophan to HFB-anhydride (HFBA) (43). Complete derivatization of tryptophan also depends on the esterification conditions (11). However, the conditions and factors required for complete acylation of tryptophan require further consideration.

In our initial studies, we acylated amino acid isobutyl esters in amounts up to 0.25 μmoles using 20 μL HFBA and 50 μL ethyl acetate (1:0). This corresponds to an HFBA–amino acid ratio of 325. However, we most often derivatized 25 μL of a standard amino acid mixture corresponding to a molar excess of about 1300-fold of the reagent with respect to a single amino acid. Under these conditions, acylation of tryptophan is incomplete. Subsequently, to simplify the reaction system, we omitted the ethyl acetate but, to maintain adequate volume in the reaction vial, used 50 μL HFBA (12). This constitutes a reagent–amino acid ratio of about 3200, and under these conditions, we obtained complete acylation. We also demonstrated clearly that acylation temperature has a significant effect on the completeness of the derivatization and suggested that it may have been responsible for the incomplete derivatization observed in another laboratory (20). Later, Moodie used reagent–amino acid ratios of 230–2300, demonstrated that the ratio is an important factor, and concluded that incomplete derivatization may be due to insufficient HFBA and the presence of ethyl acetate "rather than to acylation being carried out at too low a temperature" (43). However, the effect of temperature was not studied. Furthermore, the quality of the HFBA, which we have consistently recommended to be analytical grade, was not specified. In effect, the conclusions were not based on experimental conditions identical to those used in this laboratory.

Since our earlier experiments used an amount of HFBA which lies very close to the center of the range use by Moodie, conditions under which mono-acyl tryptophan was not observed, it must be concluded that insufficient HFBA was not responsible for incomplete acylation. In considering the proportion of HFBA to be used in derivatizing a real sample, allowance must also be made for the presence of other amino

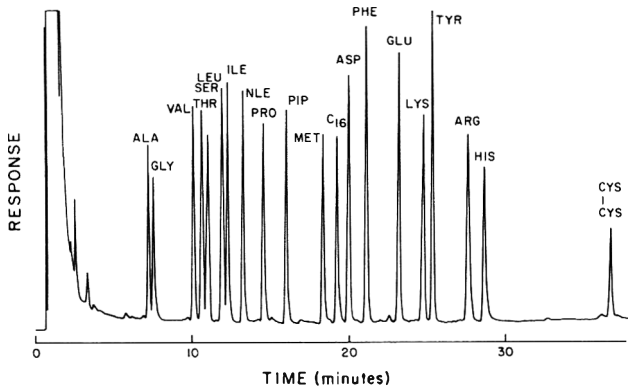

Figure 1. Chromatogram illustrating resolution of *N*-HFB amino acid isobutyl esters on 2 mm × 3 m glass column packed with 3% SE-30 on 100-120 mesh HP Chromosorb W and temperature programmed from 100°C at 4°/min.

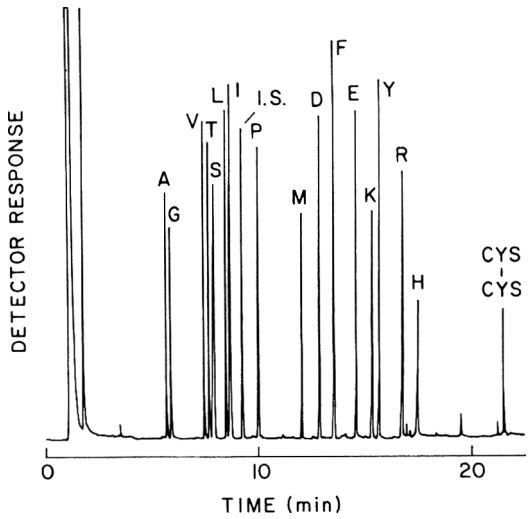

Figure 2. Chromatogram illustrating resolution of *N*-HFB amino acid isobutyl esters on 10 m × 0.2 mm OV-101-coated siloxane deactivated capillary column operated in the split mode. Oven temperature was programmed from 90 to 260°C at 8°/min. Single letters represent standard convention for amino acids. Internal standard (I.S.) was norleucine.

acids and to the polybasic nature of some amino acids. The suggestion that the presence of ethyl acetate is a factor (26) must certainly be considered, although it is more likely that impurities in this reagent, for example, peroxides (38), were responsible.

In summary, *both* the proportion of HFBA and the acylation temperature are important.

Di-HFB histidine isobutyl ester is unstable. Several methods have been used to overcome this problem but the final solution is still being sought. Reaction with ethoxyformic anhydride (EFA) to form a mixed anhydride has been suggested but is not recommended because the *N*-HFB isobutyl esters of methionine, arginine, and tyrosine are degraded by about 25, 69, and 34%, respectively (44). There is no advantage in obtaining a more reliable assay for histidine at the cost of unreliable results for 3 other amino acids and, in addition, requiring each sample to be assayed twice.

The most consistently reliable procedure is to form a mixed anhydride on-column by coinjecting the sample with the appropriate carboxylic acid anhydride (45). Following a detailed study (12) comparing the effects of several anhydrides and EFA, on-column injection with acetic anhydride has been adopted as the standard procedure in this laboratory. The chromatographic response, peak shape, and precision all compare favorably with the results obtained using EFA and the reagent is much safer to handle. The on-column reaction is most effective if an aliquot of the anhydride is drawn into the syringe, followed by an air space and then by the sample. An anhydride to sample ratio (v/v) of 0.2:1 is sufficient. The injection can be simplified by adding the anhydride directly to the sample (12) or dissolving the sample in acetic anhydride (26). Alternatively, the mixed anhydride can be formed in vitro in a third reaction.

Recommended Derivatization Procedure

The recommended procedure for preparing HFB amino acid isobutyl esters is as follows:

Dispense aliquot of sample into thick-walled vessel having Teflon-lined cap. Add aliquot of appropriate internal standard solution. Evaporate excess solvent at 50°C using stream of pure, dry nitrogen (100–200 mL/min). Add 100 μL isobutanol–3N HCl, sparge vial with nitrogen, and seal tightly. Place vial on heating block which has been preheated to 120°C. Immerse vial in oil only to level of fluid inside vial. After 5 to 10 min, remove vial and agitate contents while hot using Vortex Genie Mixer or equivalent. Replace vial on heating block and heat for remainder of a 30 min period. Remove vial and cool to room temperature before opening. Evaporate excess reagents with nitrogen and at a temperature <50°C (11).

Add 50 μL purissima grade HFBA and sparge vial with nitrogen. Seal vial tightly and heat 10 min at 150°C. Let vial cool to room temperature. Evaporate excess HFBA at room temperature with stream of nitrogen; evaporate just to dryness to avoid loss of more volatile derivatives. Dissolve residue in appropriate volume of solvent.

We have routinely used ethyl acetate as a solvent for packed column analysis, and it has been found to produce the best results using capillary columns (26). However, it may not necessarily be the most appropriate solvent for capillary column analysis because of inadequate wetting of nonpolar stationary phases (46).

Chromatography

Columns

(a) *Packed columns.*—The following discussion will concentrate on the preparation of packed columns which are adequate for most practical purposes and especially useful for a preliminary examination of a sample before analysis on a capillary column. A detailed discussion of the use of capillary columns is presented elsewhere in the report of this symposium (47).

Columns should be constructed of Pyrex glass. Silanization is not strictly necessary if the glass is cleaned thoroughly using a variety of polar and nonpolar organic solvents. If instrumental configuration does not permit direct on-column injection, a glass liner should be used in the injection system.

The best quality packings from commercial sources are not equivalent in performance, but the writer has obtained consistent results using 3% SE-30 coated on 100–120 mesh Gas-Chrom Q (12, 16, 17).

If the packing is to be prepared in-house, consistent performance will be obtained only by taking care in the selection and preparation of column materials. The best *overall* separation of *N*-HFB amino acid isobutyl esters has been obtained on a single nonpolar stationary phase (11). Essentially identical results have been obtained in this laboratory using the best available grades of SE-30, OV-1, and OV-101. The

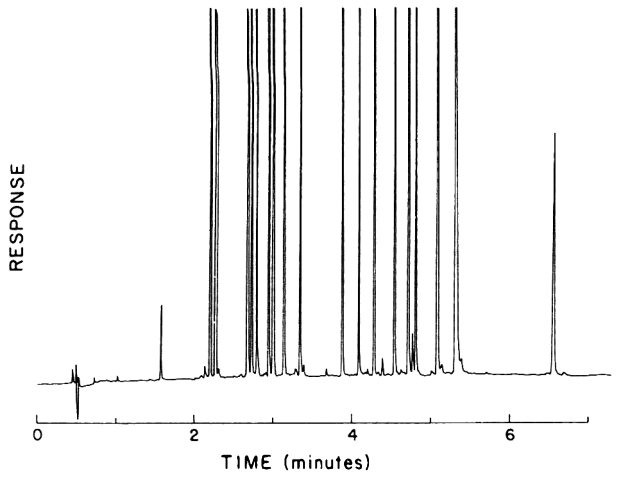

Figure 3. Chromatogram illustrating rapid analysis of standard amino acid mixture as *N*-HFB isobutyl esters on 0.2 mm × 10 m capillary column coated with OV-101 coupled to nitrogen-specific detector. Column was operated in splitless mode and programmed from 90 to 240°C at 32°/min.

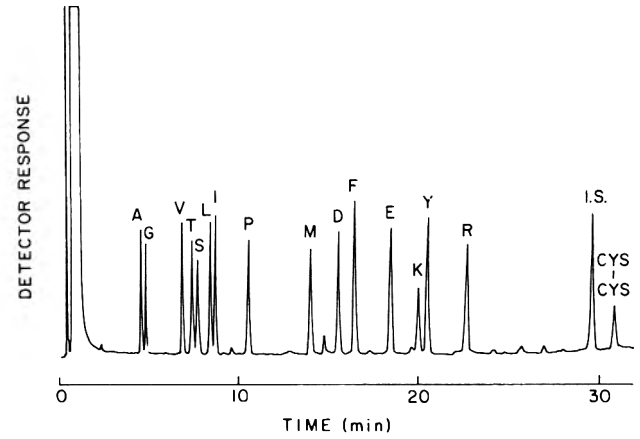

Figure 4. Chromatogram illustrating separation of *N*-HFB isobutyl amino acids using 10 m × 0.54 mm id DB-1 Megabore capillary column with helium (15 mL/min) as carrier gas. Oven temperature was programmed from 90 to 250°C at 4°/min.

results obtained do not appear to justify the additional difficulty in preparing mixed stationary phases (23) or mixed support packings (48).

The choice of support material is critical, because the resolution obtained differs on different supports even when the supports are coated with the same stationary phase. The writer has obtained the best resolution and the most consistent performance using acid-washed, high-performance Chromosorb W. Thoroughly extracting the support with chloroform and methanol before coating reduces tailing of the histidine peak.

Chromatographic resolution is directly related to support particle size. Since diatomaceous earth supports are extremely friable, the support must be sieved before coating to remove fines. Felker (38) reported that commercial packings of nominal particle size 125–149 μm may contain as much as 25% of material smaller than the specified range. Uncoated supports probably contain a substantial amount of fines also. It is equally important that packings, whether prepared in the laboratory or obtained from commercial sources, be sieved to remove agglomerated particles.

A 2 mm id × 3 m Pyrex column packed with 3% SE-30 on 100–120 mesh AW HP Chromosorb W is recommended for routine analysis of *N*-HFB amino acid isobutyl esters.

Typical chromatographic operating conditions for a packed column are: injector temperature 250°C, flame ionization detector temperature 300°C, carrier gas flow 25–30 mL/min, detector hydrogen flow 30 mL/min, and detector air flow 250–300 mL/min. Resolution of *N*-HFB isobutyl amino acids requires programming the oven temperature from 90 or 100°C to 250°C at 4°/min. Under these conditions, and using the packed column recommended in the previous section, an analysis requires about 35 min. A typical analysis is illustrated in Figure 1. Analysis time can be reduced to about 25 or 15 min by increasing the temperature program rate to 8 or 16°C/min, respectively, with little effect on precision (11, 12).

(b) *Capillary columns.* —Although packed columns are adequate for resolving the components of a mixture of proteic amino acids, the resolving power of capillary columns is useful, and at times essential, for the analysis of complex physiological samples. The theory and practice of capillary gas chromatography is described in an excellent monograph by Lee et al. (49), and specific applications to the assaying of amino acids are presented elsewhere in the report of this symposium (47). The separation of a standard calibration mixture of amino acids using a capillary column is illustrated in Figure 2.

Decreased analysis time, another potential advantage of capillary columns, is only rarely exploited. By deliberately sacrificing resolution, it is possible to reduce the time required to analyze a simple mixture such as a protein hydrolysate to less than 10 min, as illustrated in Figure 3.

Some of the advantages of a capillary column can conveniently be obtained without the need for a capillary injection system by using a wide-bore capillary (>0.5 mm). Such columns can be operated at carrier gas flows typical of packed columns (Figure 4) with comparable results, but, if makeup gas is added, can also provide the resolution of a capillary column.

Detectors

The flame ionization detector can justifiably be regarded as the standard GC detector and has been used to detect *N*-HFB isobutyl amino acids eluting from both packed (11–16, 19) and capillary columns (20–26).

The electronegativity of perfluorinated derivatives renders the HFB derivatives of amino acids particularly suitable for electron-capture detection (22–25). A 100-fold increase in sensitivity can be obtained over that obtained with the flame ionization detector (23). Chauhan and Darbre (25) obtained excellent responses for amounts of amino acid standards ranging from 1.4 pmole of tyrosine to 38 pmole of valine. In the analysis of amino acids attached to *t*-RNA, proline, lysine, and arginine were barely detectable by the flame ionization detector but were easily determined by the electron-capture detector.

The sulfur-containing amino acids have been studied in the range of 10–100 ng as the *N*-HFB isobutyl esters by using a flame photometric detector in combination with a packed column (40).

The use of a nitrogen-specific detector to assay *N*-HFB isobutyl amino acids has been reported only once (22). Chauhan and Darbre (25) obtained a linear dynamic range of 300 using a flame thermionic detector; 4 pmole of each amino acid was readily detected.

In the author's laboratory, a nitrogen-specific detector has been applied to ultramicro analysis of *N*-HFB amino acid

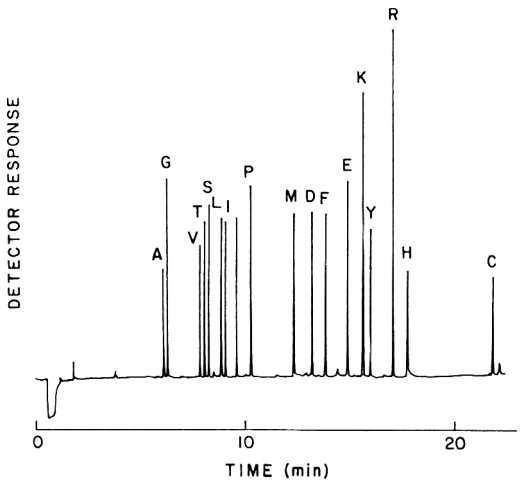

Figure 5. Chromatogram illustrating detection of *N*-HFB isobutyl amino acids using nitrogen-specific detector and 10 m × 0.2 mm OV-101 capillary column operated in splitless mode. Each peak represents 30 pmole of an amino acid.

isobutyl esters (50). In combination with a capillary column, operation in the low picomole range is entirely feasible. An analysis of a standard amino acid mixture on a 25 m fused silica capillary column coated with OV-101 and operated in the splitless mode is shown in Figure 5. Each peak represents 37.5 pmole of each amino acid. Area counts were of the order of 1000/pmole for monoamino compounds indicating a detection limit in the femtomole range.

Quantitation

Little information is available on the quantitative aspects of sample cleanup for amino acid analysis by GC (29). In most instances, cation exchange chromatography is used without specific regard to recovery. Even when internal standards are added, quality control data are seldom reported. Desgres et al. (21) added a primary internal standard to urine and plasma samples before ion-exchange treatment and a secondary internal standard before derivatization. The consistency of the ratio of the relative molar responses of the 2 standards indicated that recovery of amino acids after cleanup was quantitative. Bengtsson and Odham (23) noted that direct desalting of 100 ng amino acids on Amberlite IR-120 yielded less than 50% recovery of amino acids but that the yield with Dowex 50 was acceptable although highly dependent on the pH of the sample.

Adams (9) used internal standards and comparison with amino acid standards which were not subjected to the cleanup step to evaluate the recovery from Dowex-50. Data relating to 14 amino acids were reported; the recovery was quantitative except for histidine (91%) and arginine (79%). Similar experiments with serum, in which adsorption of amino acids by protein might occur, produced similar results. The importance of control experiments embracing all the amino acids and the application of appropriate correction factors were emphasized.

Boila and Milligan (51) reported that the recovery and precision of the recovery were related to the concentration of the NH_4OH solution used to elute amino acids from a strong cation exchange resin. Increased variability at an NH_4OH concentration equal to or greater than 2N was concluded to preclude precise quantitative measurement of the concentration of amino acids in biological samples, but this phenomenon, which should not be dismissed lightly, has not been reported by others who have used 3N NH_4OH (21–23).

Table 1. Relative molar responses of *N*-HFB isobutyl amino acids[a]

Amino acid	Reference					
	11	22	38[b]	20[c]	21[c]	22[c]
Ala	0.64	0.69	0.64	0.67	1.05	0.68
Gly	0.59	0.55	0.56	0.64	0.87	0.56
Val	0.90	0.92	0.83	0.89	1.13	0.89
Thr	0.97	0.92	0.91	0.98	0.94	0.92
Ser	0.88	0.78	0.80	0.89	0.87	0.79
Leu	1.00	1.02	1.02	1.05	0.95	1.03
Ile	1.01	0.99	0.98	1.00	1.11	0.99
Pro	0.90	0.92	0.90	0.91	0.92	0.91
Met	0.89	0.92	0.86	0.77	0.59	0.93
Asp	1.19	1.11	1.10	1.11	0.96	1.14
Phe	1.36	1.35	1.33	1.33	1.16	1.35
Glu	1.23	1.21	1.20	1.19	1.01	1.23
Lys	1.12	1.12	0.81	1.04	0.77	1.14
Tyr	1.40	1.38	1.64	1.22	1.01	1.38
Arg	1.17	0.96	1.09	0.86	0.65	1.06
His	0.94	0.83	—	0.56	0.49	0.85
Trp	1.15[d]	—	1.17	—	—	0.85
Cys | Cys	1.04	0.97	—	0.28	0.50	1.33

[a] All values relative to norleucine = 1.
[b] Calculated from original data.
[c] Data in these columns refer to capillary columns; all other data refer to packed columns.
[d] Reference 12.

Zumwalt et al. (30) obtained quantitative recovery of amino acid standards using both cation and anion exchange chromatography.

The quantitative aspects of the formation of *N*-HFB amino acid isobutyl esters have been studied in a number of laboratories (11, 12, 19–22, 38, 42, 48). The relative molar response (RMR) is the most frequently used measure of quantitative derivatization; values obtained in different laboratories using flame ionization detection have been collated previously (28), and a selection is presented in Table 1. The selection represents laboratories whose results are in substantial overall agreement and the procedures used therein appear to be the most effective in producing quantitative derivatization of all the components of a standard amino acid mixture. Although the values were obtained using different protocols, the coefficient of variation of the packed column results is less than 10% for all the amino acids except arginine, histidine, lysine, and cystine and less than 2% for leucine, isoleucine, proline, phenylalanine, and glutamic acid. The considerably greater variability in the RMR of arginine, lysine, histidine, and cystine is no doubt due to factors which have been considered above. The data obtained using capillary columns represent different injection methods and thus are not directly comparable.

Data relating to the precision of analysis of standard amino acid mixtures as the *N*-HFB isobutyl esters have been compiled previously (28). When electronic peak area integration is used, the precision compares favorably with other methods of amino acid analysis. For instance, 3 separate standards comprising proteic amino acids were analyzed in triplicate on a packed column with flame ionization detection with a precision of $<1\%$ for all except cystine (1.35%) (11). Comparable data were obtained in other laboratories for analyses embracing a variety of experimental conditions including analysis of multiple standards derivatized over the short term and, therefore, presumably involving common batches of reagents (22, 33) and the analysis of multiple standards over periods as long as 6 months (21, 28, 42). The data included

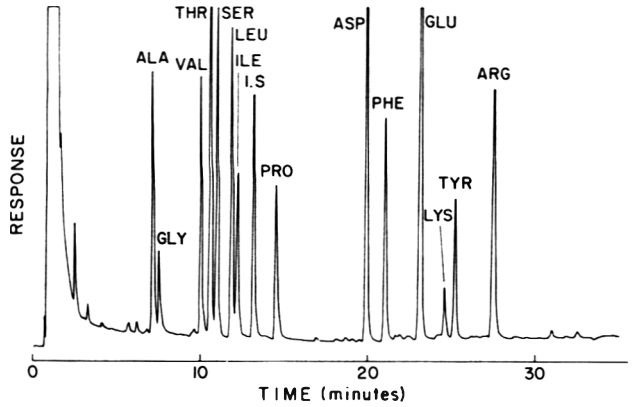

Figure 6. Chromatogram illustrating analysis of tobacco mosaic virus coat protein hydrolysate. Chromatographic conditions were as described in Figure 1.

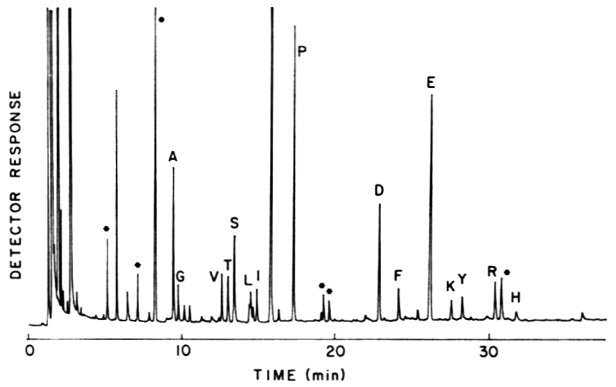

Figure 7. Chromatogram illustrating analysis of White Spruce leaf free amino acids as N-HFB isobutyl esters. Chromatographic conditions were as described in Figure 2 except that column was operated in splitless mode and temperature program rate was 4°/min.

the use of capillary columns (21, 22, 26) and different injection modes (22, 26).

Information on the precision obtainable using detectors other than flame ionization detectors is sparse, but data are available on electron-capture detectors (24, 25) and nitrogen-specific detectors (22).

Data on the accuracy of analysis of amino acids as the *N*-HFB isobutyl esters are sparse. Moodie (43, 44) presented data obtained using packed columns and flame ionization detection to analyze mixtures of known composition. The accuracy was typically within 0.6% for all the proteic amino acids except alanine (1.21%) and valine (0.78%). Similar data obtained using capillary columns (26) indicated that on-column injection is recommended for quantitative analysis. In contrast to packed column results (33), generally better precision was obtained if the samples were injected in ethyl acetate rather than acetic anhydride.

Electronic integration is recommended for peak area measurement. Quantitative peak measurement requires the use of internal standards. Norleucine, hydroxyproline, *homo*arginine, pipecolic acid, 6-methyllysine, and cycloleucine have all been used; the choice is governed by the nature of the sample being analyzed.

Applications

Analysis of Protein Hydrolysates

The results of the analysis, in the author's laboratory, of a typical protein hydrolysate by GC as the *N*-HFB isobutyl esters are illustrated in Figure 6. The tobacco mosaic virus coat protein was a highly purified preparation requiring no special preparation before hydrolysis. Other applications by the author include the determination of the compositions of heat-stable enterotoxins (17) and mustard seed storage proteins (52).

Applications of the method to protein hydrolysates in other laboratories have covered a wide range of sample matrixes. Selected examples are the analyses of the protein components of barley endosperm cell walls (53), of plasma membrane fractions from frog skeletal muscle (54), of sheep muscle and sheep urine (42), of β-lactoglobulin and casein fractions (20), and of insulin (22). Quantitative data were not always provided but the chromatograms are useful guides to the results to be expected in analyzing similar samples. Siezen and Mague (55) determined the concentration of amino acids in hydrolyzed particulate matter from different regions and depths of the Pacific Ocean. Amino acids attached to *t*-RNA have been analyzed in silk worms (25) and in rabbit reticulocytes and liver (35).

Analysis of Free Amino Acids

The proteic amino acids are of primary importance even as free amino acids and, usually, only a few nonproteic amino acids are assayed in any one sample. Before the application of *N*-HFB isobutyl amino acids to the analysis of real samples is considered, the range of biologically important amino acids which have been chromatographed as pure compounds will be reviewed.

The most extensive single study of the *N*-HFB isobutyl esters of nonproteic amino acids was conducted by Siezen and Mague (19) who examined the derivatization and chromatography on a packed column of 50 biologically important amino acids. Seventeen nonproteic amino acids could be identified and assayed in the presence of the proteic amino acids. These and other nonproteic amino acids examined in other laboratories are listed in Table 2. Their chromatographic elution properties and coeluting compounds are detailed in another report (28).

Siezen and Mague also derivatized norvaline, 2-aminoisobutyric acid, 3-aminoisobutyric acid, and 3-aminobutyric acid, but these compounds could not be sufficiently resolved from proteic amino acids on a packed column. Several compounds coeluted precisely with a proteic amino acid. These were *homo*serine with proline, 4-amino-3-hydroxybutyric acid and *S*-methylcysteine with cysteine, methionine sulfoxide with methionine, and 2,6-diaminopimelic acid with tryptophan. No peaks were observed for taurine, 6-*N*-trimethyl-5-hydroxylysine, and arginosuccinic acid. Taurine would probably lose the sulfate ester group to produce the HFB derivative of aminoethylene in a manner similar to cysteic acid (14). This derivative would probably elute with the solvent peak and thus not be observed as a reaction product.

The elution temperatures of 2,5-diaminoadipic acid, azetidine-2-carboxylic acid, *S*-ethylcysteine, *S*-ethylhomocysteine, *homo*cysteine, 2-aminoheptanoicacid, *homo*isoleucine, lanthionine, 2-aminooctanoic acid, 2-aminopimelic acid, 5-hydroxypipecolic acid, and 2-aminosuberic acid were predicted (19).

The writer has confirmed the elution characteristics of azetidine-2-carboxylic acid, *S*-ethylcysteine, *homo*cysteine, lanthionine, 2-aminopimelic acid, and 5-hydroxypipecolic acid. In addition, the following have been derivatized and chromatographed on a packed column (28): 3-chloroalanine, alliin, anthranilic acid, 3-hydroxyanthranilic acid, *p*-aminobenzoic acid, 2-amino-3-phenylbutyric acid, 2-aminocaprylic acid, 1-amino-cyclopropane-1-carboxylic acid, *allo*-cysta-

Table 2. Nonproteic amino acids analyzed as *N*-HFB isobutyl esters

Amino acid	Refs[a]	Amino acid	Refs
Adipic acid, 2-amino	a	Histidine, 1-methyl	e
Adipic acid, 2,5-diamino	a	Histidine, 3-methyl	e
β-Alanine	a b c e	Imidazole acetic acid	e
Alanine, 3-chloro	e	Imino diacetic acid	e
Alanine, 3-cyano	e	Isoleucine, *allo*	b e
Alliin	e	Isoleucine, *homo*	a
Anthranilic acid	e	Kainic acid	e
Anthranilic acid, 3-hydroxy	e	Lanthionine	a b d e
Arginine, *homo*	b–d	Leucine, cyclo	b e
Azetidine 2-carboxylic acid	a e	Levulinic acid, 5-amino	e
Benzoic acid, *p*-amino	e	Lysine, 5-hydroxy	a e
n-Butyric acid, 2-amino	a–d	Lysine, 6-methyl	b d
Butyric acid, 2-amino, 3-phenyl	e	Methionine, 2-methyl	e
n-Butyric acid, 3-amino	a	Methionine sulfone	b e
n-Butyric acid, 4-amino	a b e	Methionine sulfoxide	a e
n-Butyric acid, 2,4-diamino	a	Norleucine	a c d e
n-Butyric acid, 3-OH, 4-amino	a	Norvaline	a
iso-Butyric acid, 2-amino	a	Octanoic acid, 2-amino	a
iso-Butyric acid, 3-amino	a b	Octanoic acid, 6-amino	e
Caprylic acid, 2-amino	e	Ornithine	a–e
Citrulline	a e	Penicillamine	e
Citrulline, *homo*	e	β-Phenylalanine	e
Creatine	e	Phenylalanine, 3,4-diOH	a c d e
Cyclopropane, 1-amino, 1-carboxy	e	Pimelic acid, 2-amino	a e
Cystathionine	a b d e	Pimelic acid, 2,6-diamino	a
Cystathionine, *allo*	e	Pipecolic acid	a c e
Cysteic acid	a e	Pipecolic acid, 5-hydroxy	a e
Cysteine, *S*-2-aminoethyl	e	Proline, 4-hydroxy	b–e
Cysteine, *S*-carboxymethyl	c e	Proline, 3,4-dihydroxy	a
Cysteine, *S*-ethyl	a e	Salicylic acid, 4-amino	e
Cysteine, *S*-ethyl *homo*	a e	Sarcosine	a e
Cysteine, *S*-methyl	e	Serine, cyclo	e
Cysteine, *homo*	a b	Serine, *homo*	a
Cystine, *homo*	b e	Serine, iso	e
Djenkolic acid	e	Suberic acid, 2-amino	a
Glutamic acid, 4-carboxy	e	Threonine, *allo*	c e
Guanidoacetic acid	e	Tryptophan, 5-hydroxy	e
Heptanoic acid, 2-amino	a	Tyrosine, 3-iodo	a
Hexanoic acid, 6-amino	c	Tyrosine, 3,5-diiodo	a

[a] References: a = Siezen & Mague (19); b = Desgres et al. (21); c = Chauhan et al. (22); d = Chauhan & Darbre (25); e = MacKenzie (28).

thionine, *S*-2-aminoethylcysteine, *S*-methylcysteine, *homo*cystine, djenkolic acid, guanidoacetic acid, 1-methylhistidine, 3-methylhistidine, imino diacetic acid, *allo*-isoleucine, kainic acid, cycloleucine, 5-aminolevulinic acid, 2-methylmethionine, 6-aminooctanoic acid, penicillamine, β-phenylalanine, 4-hydroxyproline, cycloserine, *iso*-serine, *allo*-threonine, and 5-hydroxytryptophan.

Chauhan and Darbre (22, 25) and Desgres et al. (21) used capillary columns to chromatograph many of the amino acids mentioned above. *Homo*arginine and 6-methyllysine were added to the list of amino acids studied using packed columns (21, 22). The superior resolution of a capillary column enabled the resolution of 3-aminoisobutyric acid and valine, methionine sulfone and tyrosine, and *allo*-isoleucine and isoleucine (21). *S*-Carboxyethylcysteine and kynurenine appear to have been studied only by Moodie and Burger (26) and Pearce (20), respectively. Larsen and Thornton (42) optimized the derivatization of 3-methylhistidine.

Citrulline and ornithine are indicated to coelute, but this is because citrulline is degraded to ornithine during derivatization. *Homo*serine produces 2 peaks (19). Cysteic acid loses the sulfate ester group either during derivatization or chromatography to produce dehydro-alanine. The degradation can be used to good effect by increasing the injector temperature so that the conversion is quantitative, thus allowing cysteic acid to be assayed (14).

There has been no definitive study of the resolution of nonproteic amino acids using capillary columns comparable to the packed column study of Siezen and Mague (19). A capillary column would probably resolve many of the amino acids which coelute on a packed column, and the definition of the retention times would assist the identification of unknown compounds in physiological samples.

A capillary column analysis of the free acids present in White Spruce leaves is illustrated in Figure 7. Apart from the relative abundance of 4-aminobutyric acid, the nonproteic amino acids constitute a relatively small proportion of the total sample. Although not indicated, β-alanine, pipecolic acid, and ornithine were readily identified.

The free amino acids in a basic fraction of pea xylem sap contain predominantly aspartic acid, with *homo*serine and glutamic acid being the next most abundant components. A capillary column analysis, coupled with a nitrogen-specific detector, also permits easy identification of γ-aminobutyric acid, hydroxyproline, and ornithine (Figure 8).

Although practical applications of analysis of free amino acids as *N*-HFB isobutyl esters are not yet numerous, a few examples will demonstrate an interesting diversity and hence the flexibility of the method. Thomas and Dawson (56) studied the relationship between iron- and energy-limited growth during phased cultivation of *Candida utilis* and concluded that changes in composition of the free amino acid pool of the yeast during the cell cycle depended on the type of nutrient limitation. Ferguson (57) found that ingestion of dissolved amino acids significantly elevated the tissue-free amino acid pool of the starfish, *Echinaster*. Greenaway and Ward

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Analysis of Amino Acids by Gas Chromatography as the *N*-Trifluoroacetyl *n*-Butyl Esters

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This presentation describes amino acid analysis with the gas chromatographic method and experimental conditions using the *N*-trifluoroacetyl *n*-butyl ester derivatives; the study we describe here was undertaken to compare gas chromatographic (GC) and ion-exchange chromatographic (IEC) analyses of amino acids in hydrolysates of 9 diverse sample types to gain insight into effects of these 2 chromatographic methods of analysis on variation in amino acid results. Our study showed that values for samples prepared by 2 separate laboratories using the same procedure were generally in good agreement when all of the hydrolysates were analyzed by a single laboratory using a single method of analysis. To compare results from gas chromatography with those from ion-exchange chromatography analyses were performed by 2 different laboratories on the same hydrolysates and on different hydrolysates prepared by the same method by both laboratories. The data demonstrate that GC and IEC can be expected to yield essentially identical results when applied to the same hydrolysate. Agreement is so close that interlaboratory differences in hydrolysate preparation of the same sample contribute as much to variation in amino acid results as does the method of analysis, a fact which should be noted in planning collaborative studies.

The acylated amino acid esters are the most frequently used class of derivatives for amino acid analysis by gas chromatography. Our research in this area has mainly focused on the *N*(*O*,*S*)-trifluoroacetyl (TFA) *n*-butyl ester derivatives first introduced by Zomzely et al. (1). In 1965, Lamkin and Gehrke (2) published the first in a series of reports on the development of a quantitative, reliable, and practical method for analysis of amino acids in a wide range of matrixes. The *N*(*O*,*S*)-TFA *n*-butyl esters were the subject of studies by Gehrke and coworkers on esterification (3), acylation (4), and chromatographic separation (5–8), with these developments leading to a number of applications. Zumwalt et al. (4) reported the use of the *N*-TFA *n*-butyl esters for analysis of free amino acids in urine and serum and in hydrolysates of corn grain and soybean oil meal, and they described a microscale method for derivatization of small sample quantities (9). Zumwalt et al. (10) reported on the development of a solvent-venting system which allowed the injection of 50–100 μL sample solution while preventing the solvent and acylating reagent from traversing the analytical column, and Zumwalt et al. (9) described the application of the gas chromatographic *N*-TFA *n*-butyl ester method to the analysis of lunar samples returned by Apollo 11 and 12 missions. Gehrke et al. (11–14) and Rash et al. (15) used the *N*-TFA *n*-butyl esters for analysis of lunar samples returned by Apollo flights 11–17 and reviewed the roles of gas chromatography (GC) in lunar organic analysis (16). Kaiser et al. (17) described protein hydrolysis studies of ribonuclease, fish meal, and corn, comparing 145°C, 4 h hydrolysis with and without the aid of sonication for oxygen removal, and using GC analysis of the *N*-TFA amino acid *n*-butyl esters. Comparisons were also made with hydrolysis at 110°C, 21 h, with the conclusion that the 145°C, 4 h hydrolysis procedure gave results in good agreement with the 110°C, 21 h procedure. Average relative standard deviations of the GC analyses were reported to be 1.8% for ribonuclease, 2.7% for fish meal, and 3.4% for corn.

The *N*-TFA *n*-butyl ester derivatives have found use in a variety of research areas; e.g., Amico et al. (18) used them for determinations of the free amino acid content of marine algae and reported relative molar responses of 16 protein and 30 nonprotein amino acids. Sakamoto et al. (19, 20) used the *N*-TFA *n*-butyl esters for analysis of unusual amino acids present in hydrolysates of chemically modified proteins, specifically *S*-alkylcysteines, *O*-alkyltyrosines, N^{ϵ}-alkyllysines, and N^{ϵ},N^{ϵ}-dialkyllysines.

In 1985, Sakamoto et al. (21) used the *N*-TFA *n*-butyl ester derivatives to identify the reaction products of protein fibers with alkyl glycidyl ethers. After silk fibers were treated with methyl-, ethyl-, isopropyl-, and alkyl glycidyl ethers and subjected to acidic hydrolysis, the hydrolysates were derivatized to the *N*-TFA *n*-butyl esters and analyzed by gas chromatography/mass spectrometry. Sarkar and Malhotra (22) used the *N*-TFA *n*-butyl esters for analysis of amino acids in pine needle extracts.

Nagy and Hall (23, 24) studied the GC separation of the *N*-TFA *n*-butyl esters and recommended the use of 2 columns for the separation of the 20 amino acids; they used a

Accepted June 9, 1986.
Presented at the Symposium on Chromatography of Amino Acids, 99th AOAC Annual International Meeting, Oct. 27–31, 1985, Washington, DC.
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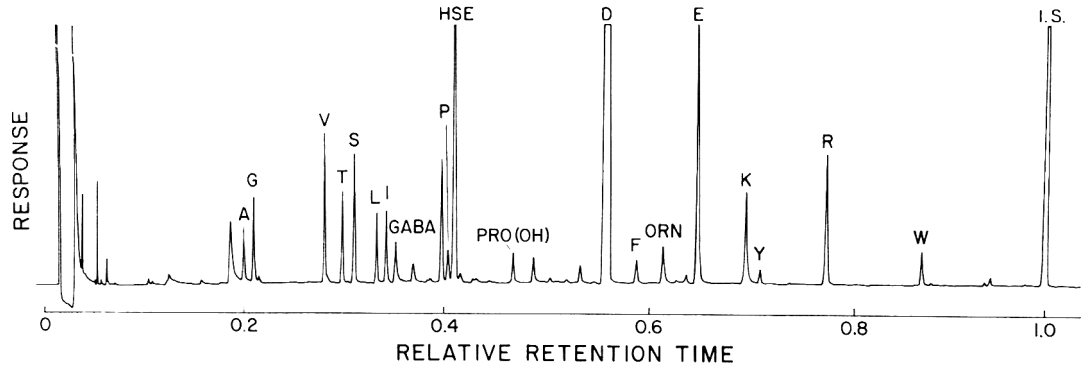

Figure 8. Chromatogram illustrating analysis of free amino acids in pea xylem sap as *N*-HFB isobutyl esters using 15 m × 0.25 mm bonded SE-30 capillary column operated in splitless mode. Oven temperature was programmed from 90 to 260°C at 4°/min.

(58) concluded that sulfur amino acids are not directly involved in the mercury resistance of certain fungi. The amino acids of major importance were cystathionine, 4-aminobutyric acid, hydroxyproline, and ornithine. 2-Amino adipic acid, β-alanine, and 2-aminobutyric acid were present but in amounts less than 1% of the total amino acid pool. The in vitro release of alanine, glycine, serine, aspartic acid, glutamic acid, ornithine, and citrulline by rumen papillae has been shown to depend on the substrate (59). Rhodes et al. (60) assayed the assimilation of $^{15}NH_4$ in *Lemna minor* L. by gas chromatography mass/spectrometry (GC/MS), monitored specific fragment ions, and concluded that the ratio of selected ion pairs could be "used to calculate ^{15}N abundance with an accuracy of ±1 atom % excess ^{15}N using samples containing as little as 30 picomoles of individual amino acids." Lindqvist and Maenpaa (35) determined the free amino acids in rabbit liver.

The advantages provided by the resolution of capillary columns have been used to good effect for the analysis of complex physiological samples. The *N*-HFB amino acid isobutyl esters were first separated on a support-coated capillary column by Pearce, who analyzed urine and plasma to diagnose human genetic disorders (20). Others have since used capillary columns to assess the physiological effects of deep sea diving (25), to determine urinary amino acids (21, 22), to estimate the excretion of free amino acids from microbial populations in natural waters (23, 24), and to assay the amino acid composition of fish muscle protein (26).

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Silar 10C column for separation of all amino acids except histidine, arginine, and cystine, for which they used a mixed phase column. They also studied the response of histidine on silicone mixed-liquid phase columns and confirmed that the response of histidine as the diacyl derivative was optimum when excess TFAA was injected. Matucha and Smolkova (25) used these derivatives for determining the purity of carrier-free preparations of amino acids biosynthetically labeled with ^{14}C and for determining the specific activity of these labeled materials. Quantitative differences in the amino acid composition of heart muscle were reported by Gabrys and Konecki (26), who used GC analysis of the *N*-TFA *n*-butyl esters.

Successful gas chromatographic methods for amino acid analysis have been developed which provide equivalent, complementary, and additional alternatives to the classical ion-exchange chromatographic separation of amino acids followed by post-column ninhydrin derivatization and spectrophotometric detection. These GC methods have been developed to overcome certain limiting characteristics of the conventional IEC-ninhydrin detection analyzers, including high cost, moderate sensitivity, long analysis times, and lack of versatility for doing other types of analyses. Although reports in the literature compare GC and IEC analyses of specific types of samples, and those data do, in general, agree well, GC–IEC comparisons have not been reported to the extent that might be expected (27), given the widespread interest and use of these techniques over some 20-plus years. This is especially noticeable because the 2 techniques differ considerably in separation mechanisms, methods of sample preparation, requirement for sample derivatization at different points in the procedures, and means of detection.

Our 3-volume text on amino acid analysis by gas chromatography extensively covers both packed column and capillary GC analysis of amino acids, with chapters contributed by 15 research groups from 12 countries (28). We present, in this symposium paper and in the previous reference, a broad range of comparisons, from orchard leaves to pure proteins, and a representative sampling is presented in the following citations.

Pellizzari et al. (29, 30), Lewis and Waterhouse (31), and Zumwalt et al. (4) have compared GC and IEC analyses of free amino acids in blood plasma, reporting good agreement between the 2 methods of analysis. March (32) compared GC and IEC analyses of insulin and α-chymotrypsin hydrolysates, as well as hydrolysates of protein fractions obtained during purification of cytochrome f from pea leaves, again obtaining good agreement. Kirkman (33) compared the determination of protein amino acids in plant material by GC and IEC analysis, and Gehrke et al. (34) reported amino acid analysis comparisons for soybean meal, corn grain, serum, and urine, with good agreement of data obtained by the 2 methods.

In a critical review, Labadarios et al. (35) observed that despite the added time required to prepare volatile derivatives, GC analysis of amino acids offers advantages in capital and operating costs, shorter elution time, and improved precision and accuracy as compared to amino acid analyzers. The relative standard deviations of peak areas obtained from amino acid analyzer analyses of amino acid standard mixtures are generally quoted to be ±2%, whereas equivalent data of below 1% are routinely possible using GC analysis (35). The sensitivity threshold of ninhydrin detection is claimed to be about 10 ng, and about 1 ng if fluorescence detection of *o*-phthalaldehyde amino acid derivatives is used. GC analysis with electron capture detection offers sensitivity to the 10–100 pg level, and if it is coupled with mass spectrometry, sensitivity can be increased still further.

Labadarios et al. (35) noted the increasing use of gas chromatography in amino acid analysis of physiological fluids in their retrospective literature search, citing 36 references which contained details on purification procedures of the protein amino acids from biological fluids prior to GC analysis.

This presentation describes amino acid analysis with the GC method and experimental conditions using the *N*-TFA *n*-butyl ester derivatives. The study we describe here was undertaken to compare GC and IEC analyses of amino acids in hydrolysates of 9 diverse sample types to gain insight into effects of these 2 chromatographic methods of analysis on variation in amino acid results. We felt this information would be of considerable value to laboratories that would like to participate in comparative or collaborative studies of amino acid analysis, and it would provide GC–IEC comparative data on specific amino acids in different sample matrixes.

Also, we describe a comparison of hydrolysates of the 9 different sample types prepared in 2 different laboratories, but analyzed by ion-exchange chromatography in a single laboratory. Our study showed that values for samples prepared by 2 separate laboratories using the same procedure were generally in good agreement when all of the hydrolysates were analyzed by a single laboratory using a single method of analysis. This information is of considerable value in the design and execution of collaborative studies on amino acids analysis.

Having obtained some insight into the level of between-laboratory variation which can be expected in hydrolysate preparation (36), we compared the effects of method of analysis (GC and IEC) on amino acid values in hydrolysates of diverse sample types. Two types of comparisons are reported: first, a comparison of GC analyses by one laboratory with IEC analyses performed by a different laboratory on the same hydrolysates; and second, comparison of GC and IEC analyses performed by the 2 laboratories on different hydrolysates, each prepared by the same method in both laboratories.

Amino acid analysis by capillary gas chromatography is described in other presentations in this symposium, and this approach will become more widely used with the increasing use of bonded-phase fused silica capillary columns.

Packed column GC analysis is a straightforward, versatile, yet powerful analytical tool that is widely available at moderate cost, and has therefore found wide use in many different areas of research concerning amino acids.

Experimental

Preparation of Hydrolysates

Single hydrolysates of 9 sample types (see Table 1, ref. 36) were prepared in the Experiment Station Chemical Laboratories (ESCL) at the University of Missouri and by Analytical BioChemistry Laboratories, Inc. (ABC), a commercial laboratory located in Columbia, MO. Both laboratories used the 145°C, 4 h hydrolysis procedure (37), with ESCL using sealed ampoules and ABC using glass tubes with Teflon-lined screw caps.

Apparatus

Hewlett-Packard Model 5880 and Varian Model 2100 gas chromatographs with flame ionization detectors were used. Digital integration was used for determining peak areas. Adsorbent trap filters containing a combination of molecular

sieve 5A, charcoal, and Drierite for the hydrogen, air, and nitrogen lines were obtained from Regis Chemical Co. (Morton Grove, IL) and Supelco, Inc. (Bellefonte, PA).

An ultrasonic cleaner was used to mix the samples after the addition of reagents (Mettler Electronics, Anaheim, CA).

A Sol-Vent system (Pat. No. 3,881,892) was used in some cases which allowed the injection of large amounts (10–100 μL) of derivatized sample solution onto the analytical column.

Two constant-temperature oil baths, set at 100 and 150°C ± 2°, were used for esterification and acylation, respectively.

Glass drying tubes, inverted form, with ȿ 24/40 inner joints at one end were obtained from Fisher Scientific (St. Louis, MO), as were 125 mL, flat-bottom ȿ 2/40, short-neck flasks.

Solvents were removed from the samples with a CaLab rotary evaporator, "cold finger" condenser (all-glass and PTFE) (CaLab Equipment Co., Oakland, CA) and a Model 1400 Welch Duo-Seal vacuum pump (Fisher Scientific).

Ion-exchange columns, 15 × 150 mm, containing porous glass frits were obtained from Arthur H. Thomas Co. (Philadelphia, PA) for cation-exchange cleanup of samples.

Morton flasks (ribbed round-bottom flasks) were purchased from Scientific Glass Blowing Co., Houston, TX.

Pyrex 16 × 75 mm or 13 × 100 mm glass, screw-top culture tubes (Corning No. 9826) with Teflon-lined caps were used as reaction vessels for the acylation reactions. Microreaction vials were made by the glass blowing service, University of Missouri, from 13 × 100 mm glass culture tubes. Teflon-lined screw caps were used to close the vials.

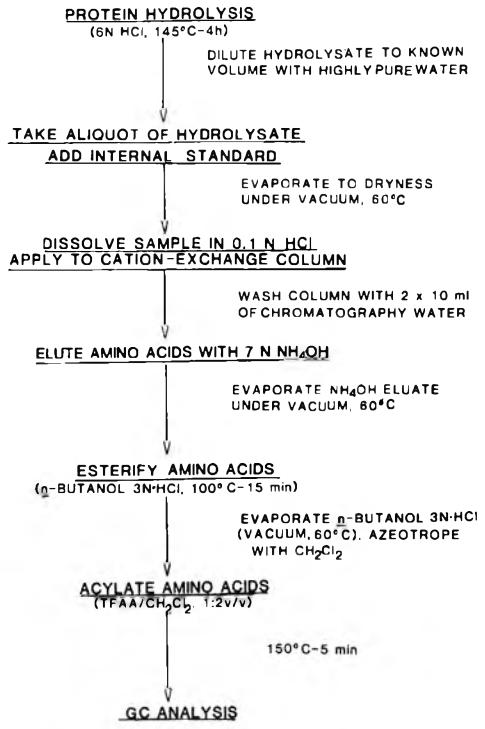
Reagents and Materials

n-Butanol was ACS reagent grade from Fisher Scientific, dichloromethane was "nanograde" quality from Mallinckrodt, and trifluoroacetic anhydride (TFAA) was obtained from Regis Chemical Co., Morton Grove, IL. The internal standard *trans*-4-aminocyclohexanecarboxylic acid (tranexamic acid) was obtained from Aldrich Chemical Co., Milwaukee, WI. Pure anhydrous HCl gas was generated by the slow addition of reagent grade HCl into concentrated H_2SO_4. The HCl gas was passed through 2 drying towers containing concentrated H_2SO_4, then slowly bubbled into *n*-butanol until 3N in HCl. The normality was checked by titration with standardized bases; also, *n*-butanol 3N HCl reagent was purchased from Regis Chemical Co.

Preparation of Chromatographic Column Packings

Packing I.—Ethylene glycol adipate (EDA), stabilized grade (Supelco, Inc.); 0.65% (w/w) on 80–100 mesh Chromosorb W(AW) (Applied Science, Deerfield, IL); acetonitrile, anhydrous, nanograde (Mallinckrodt).

For preparation of 30 g packing, 29.805 g Chromosorb W(AW) is weighed into 500 mL Morton flask, then acetonitrile is added until liquid level is ca ⅛ in. above Chromosorb W. Into clean 100 mL beaker, 0.195 g EGA is weighed, then ca 20–25 mL acetonitrile is added, and EGA is dissolved with the aid of an ultrasonic bath. EGA solution is then added to same Morton flask, and the flask is placed on a rotary evaporator maintained in slow rotation (all-glass or PTFE). Solvent is slowly removed under partial vacuum at room temperature for ca 1 h. When Chromosorb is still damp, but does not adhere to side of flask, flask is removed from evaporator and inside walls are washed with 3–4 mL acetonitrile. Flask is placed on the evaporator and solvent is removed with slow rotation under partial vacuum. When packing is only slightly damp, the vacuum is increased, and the flask is immersed in a 60°C water bath with continued rotation until solvent is completely removed. At this point, no EGA packing should adhere to inner wall of the flask.



Scheme 1. Protein hydrolysis, cleanup, and derivatization.

To prepare the chromatographic column, packing is poured into clean, dry 1.5 m × 4 mm id (or 2 mm id) glass columns with gentle tapping. Silanized glass wool plugs are placed in each end to hold packing in place.

Packing II.—1.0% (w/w) OV-7 + 0.75% (w/w) SP-2401 on 100-120 mesh Gas-Chrom Q (Applied Science); acetone, anhydrous, nanograde (Mallinckrodt).

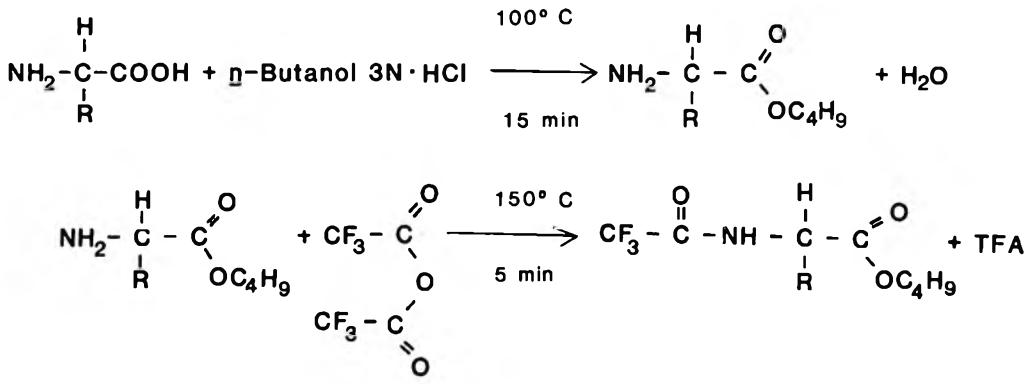
For preparation of 30 g packing, 29.475 g Gas-Chrom Q is weighed into 500 mL Morton flask, then acetone is added until liquid level is ca ⅛ in. above Gas-Chrom Q. Into clean, dry, 100 mL beakers, 0.300 g OV-7 and 0.225 g SP-2401 are weighed, then ca 20–25 mL acetone is added and liquid phase is dissolved. Each liquid phase solution is then added to Morton flask, and solvent is removed in the same manner as described above. Dried column packing is placed in 1.5 m × 4 mm id glass columns, and silanized glass-wool plugs are inserted.

Schematic Presentation of the Technique

The technique for analysis of the amino acid content of proteinaceous materials by gas chromatography of the amino acid *N*-TFA *n*-butyl esters is shown in Scheme 1. The major steps are sample hydrolysis, cation-exchange cleanup of the hydrolysate (if required), quantitative derivatization of the amino acids to the *N*-TFA *n*-butyl esters (Scheme 2), and separation and quantitation of the derivatives using the dual-column system with ethylene glycol adipate and mixed silicone liquid phases.

Sample Derivatization: Esterification and Acylation

(a) An aliquot containing ca 10 mg protein hydrolysate is placed in a 125 mL round-bottom flask. The internal standard (tranexamic acid) is added by addition of a 5.00 mL



Scheme 2. Derivatization of amino acids to *N*-trifluoroacetyl *n*-butyl esters.

aliquot of a solution containing 0.10 mg/mL of tranexamic acid in 0.1N HCl, and the sample solution is then evaporated to dryness with a rotary evaporator under vacuum in a 60°C water bath.

(b) Approximately 15 mL *n*-butanol·3N HCl (1.5 mL/mg amino acids) is added, a magnetic stirring bar is placed in the esterification flask, and the flask is closed with a glass drying tube containing Drierite. Two lead rings are placed over the neck of the flask for stability.

(c) The flask is placed in an ultrasonic bath for 1 min and then into a 100°C ± 2° oil bath on a magnetic stirring hot plate for 15 ± 1 min. The solution is stirred during the 15 min esterification period.

(d) Following esterification, the flask is allowed to cool, and *n*-butanol·3N HCl is removed under vacuum in a 60°C ± 5° water bath.

(e) Approximately 5 mL dry methylene chloride is added to remove azeotropically any remaining water and is then removed under vacuum in the 60°C water bath. The flask is then allowed to cool.

(f) Approximately 2 mL dry methylene chloride and 1 mL TFAA are added to the flask and mixed. Sample is then transferred to two 8 mL Pyrex glass culture tubes and capped with PTFE-lined screw caps (one sample as reserve).

(g) Sample is acylated at 150°C ± 2° for 5 min and not exceeding 6 min. Following acylation, derivatized samples are placed in a freezer at 0° until chromatography is performed, although prompt analysis is recommended.

Amino Acid Analysis of Protein Hydrolysates

The GC analyses were performed by ABC using the method described above. The hydrolysates of the 9 sample types were prepared singly by ABC and were analyzed in triplicate, with 3 independent aliquots of each hydrolysate taken through the cleanup, derivatization, and analysis procedures. The GC value of each amino acid for each sample type is the mean of these 3 determinations.

To compare GC and IEC analyses of the same hydrolysate, aliquots of the single hydrolysates which ABC had prepared and analyzed by GC were provided to ESCL for IEC analysis with a Beckman 121 M amino acid analyzer.

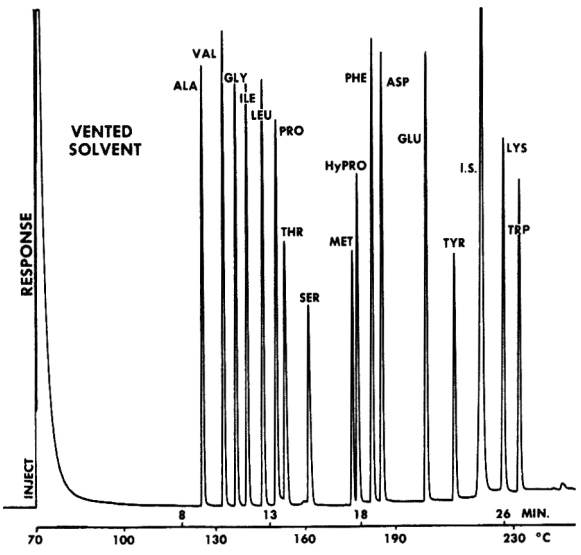


Figure 1. GC analysis of standard amino acid mixture. Standard mixture, 200 μg each amino acid; final acylation volume, 3 mL; injected, 15 μL (ca 1.0 μg of each amino acid injected); temperatures: initial 70°C, injection port 180°C, final 230°C; solvent vent time, 15 s; program rate, 6°/min; attenuation, 32 × 10^{-10} AFS. Column: 0.65 w/w% EGA on 80-100 mesh Chromosorb W(AW), 1.5 m × 4 mm id glass. Precolumn: 1.0 w/w% OV-17 on 80-100 mesh Chromosorb G(HP), 4 in. × 4 mm id, *n*-butyl stearate as internal standard.

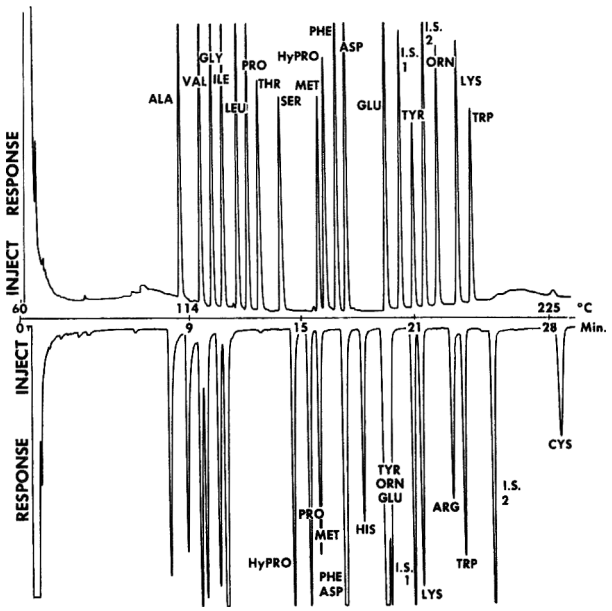


Figure 2. Simultaneous GC separation of protein amino acids. Sample, *N*-trifluoroacetyl *n*-butyl esters; injected, ca 0.6 μg of each; attenuation, 1 × 10^{-10} AFS; temperatures: initial 60°C, final 225°C; program rate, 6°/min. Top column: 0.65 w/w% stabilized grade EGA on 80-100 mesh Chromosorb W(AW), 1.5 m × 4 mm id glass; internal standard 1, tranexamic acid; internal standard 2, *n*-butyl stearate. Bottom column: 2.0 w/w% OV-17 + 1.0 w/w% OV-210 on 100-120 mesh Supelcoport, 1.5 m × 4 mm id glass.

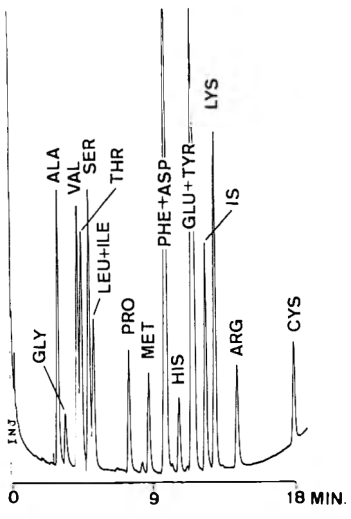


Figure 3. GC analysis of His, Arg, and Cys. Sample, ribonuclease. Column: mixed phase, 1% OV-7 + 0.75% SP-2401 on 100-120 mesh Gas-Chrom Q, 1.8 m × 4 mm glass.

Second, ESCL prepared a separate set of the 9 hydrolysates and analyzed each in duplicate with IEC. These IEC values were compared with the GC analyses of the ABC-prepared hydrolysates to observe between-laboratory variations resulting from both hydrolysate preparation and different methods of analysis.

Results and Discussion

Chromatographic Columns

The protein amino acid *N*-TFA *n*-butyl esters were separated on the ethylene glycol adipate and mixed-silicone phase columns, with the EGA column separating all the amino acids except the very polar histidine and arginine and the highest molecular weight amino acid, cystine. Earlier, we had reported (17) the use of a mixed phase column composed of 2.0% OV-17 + 1.0% OV-210 on Gas-Chrom Q for the analysis of these 3 amino acids. As a result of more recent studies,

Table 1. Average percent differences between IEC and GC analyses of amino acids of 9 different sample types[a,b]

Amino acid	Percent difference by GC and IEC	
	Same hydrolysate[c]	Different hydrolysates[d]
Ala	2.1	5.4
Val	5.0	10.1
Gly	2.2	5.7
Ile	4.7	6.9
Leu	3.4	7.2
Pro	2.1	5.0
Thr	2.3	2.3
Ser	3.3[e]	4.7[e]
Phe	2.4	6.3
Asp	4.9	6.1
Glu	3.3	7.1
Tyr	3.6	7.9
Lys	4.7	6.9
His	5.9	6.3
Arg	4.6	6.9
Average	3.6	6.3

[a] Average percent difference between IEC and GC values for all 9 sample types (see Table 1, ref. 36).
[b] 145°C, 4 h, sealed ampoule hydrolysis.
[c] Three Independent GC analyses of a single hydrolysate compared with a single IEC analysis of the same hydrolysate.
[d] Single IEC analyses of single hydrolysates of each sample type, prepared and analyzed at ESCL, compared to 3 independent GC analyses of a single hydrolysate of each sample type prepared and analyzed at ABC.
[e] Average of 5 lowest serine difference values.

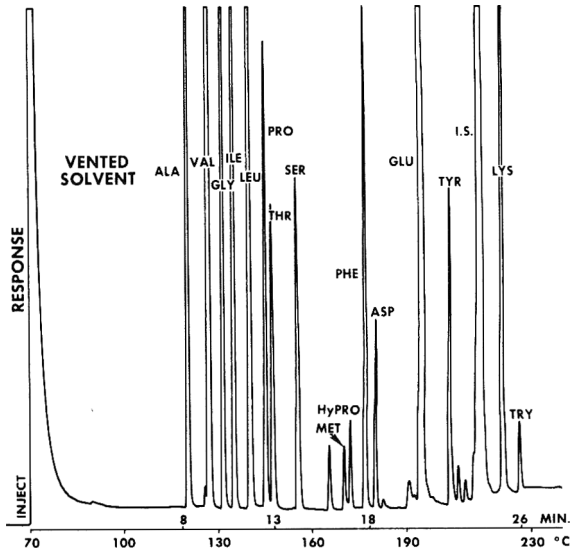


Figure 4. GC analysis of bovine blood plasma. Sample, 10 mL plasma deproteinized with 40 mL 1% picric acid, cation-exchange cleaned; final acylation volume, 2 mL; injected, 5 μL (ca 5 μg total amino acids injected); temperatures: initial 70°C, injection port 180°C, final 230°C; solvent vent time, 25 s; program rate 6°/min; attenuation, 8 × 10^{-10} AFS. Column: 0.65 w/w% EGA on 80-100 mesh Chromosorb W(AW), 1.5 m × 4 mm id glass. Precolumn: 1.0 w/w% OV-17 on 80-100 mesh Chromosorb G(HP), 4 in. × 4 mm id, *n*-butyl stearate as internal standard.

we have found a mixed phase consisting of 1% OV-7 + 0.75% SP-2401 on 100–120 mesh Gas-Chrom Q to be well suited for analysis of histidine, arginine, and cystine. Figure 1 illustrates the EGA separation of a standard amino acid mixture, and Figure 2 shows the simultaneous separation of the protein amino acids with EGA and the earlier OV-17 + OV-210 mixed phase columns. The analysis of histidine, arginine, and cystine in a ribonuclease hydrolysate is shown in Figure 3. Analysis of bovine blood plasma with the EGA column is demonstrated in Figure 4, with solvent venting.

GC and IEC Results on Hydrolysates of Diverse Matrixes

Same hydrolysate.—To compare GLC and IEC amino acid analyses, triplicate GC analyses of hydrolysates of 9 sample

Table 2. Sum of 15 amino acids analyzed by IEC and GC[a,b,c]

Sample	Same hydrolysates[d]		Percent difference	Different hydrolysates[e]		Percent difference
	IEC	GC		IEC	GC	
Soybean meal	43.97	43.96	0.0	44.93	43.96	−2.2
Poultry feed	18.65	18.08	−3.1	18.53	18.08	−2.4
Fish meal	55.12	52.83	−4.2	56.80	52.83	−7.0
Wheat	13.57	13.42	−1.1	13.97	13.42	−3.9
Pruteen	59.24	58.26	−1.7	60.52	58.26	−3.7
NBS Orchard Leaves	13.18	13.44	+2.0	14.19	13.44	−5.3
NBS Bovine Liver	52.41	53.26	+1.6	59.30	53.26	−10.2
Egg white	78.85	77.90	−1.2	86.00	77.90	−9.4
Ribonuclease	90.83	88.67	−2.4	89.85	88.67	−1.3
Average			1.9			5.0

[a] Methionine, cystine, and tryptophan excluded.
[b] 145°C, 4 h, sealed ampoule hydrolysis.
[c] Values are w/w%.
[d] Three independent GC analyses of a single hydrolysate compared with a single IEC analysis of the same hydrolysate.
[e] Single IEC analyses of single hydrolysates of each sample type, prepared and analyzed at ESCL, compared to 3 independent GC analyses of a single hydrolysate of each sample type prepared and analyzed at ABC.

Table 3. Comparison of IEC and GC analyses of the same protein hydrolysates[a,b]

Amino acid	Soybean meal, w/w%				Poultry feed, w/w%				Fish meal, w/w%			
	IEC[c]	GC[d]	Diff.	% Rel. diff.	IEC	GC	Diff.	% Rel. diff.	IEC	GC	Diff.	% Rel. diff.
Ala	2.04	2.05	+0.01	0.5	1.10	1.08	−0.02	1.8	3.92	3.86	−0.06	1.5
Val	2.34	2.27	−0.07	3.0	1.02	0.97	−0.05	4.9	3.11	2.92	−0.19	6.1
Gly	2.02	2.02	0.00	0.0	0.94	0.92	−0.02	2.1	4.51	4.43	−0.08	1.8
Ile	2.14	2.29	+0.15	7.0	0.84	0.87	+0.03	3.6	2.55	2.64	+0.09	3.5
Leu	3.67	3.59	−0.08	2.2	1.81	1.73	−0.08	4.4	4.48	4.25	−0.23	5.1
Pro	2.39	2.38	−0.01	0.4	1.30	1.27	−0.03	2.3	2.95	2.86	−0.09	3.1
Thr	1.77	1.86	+0.09	5.1	0.71	0.73	+0.02	2.8	2.61	2.54	−0.07	2.7
Ser	—	—	—	—	—	—	—	—	2.31	2.33	+0.02	0.9
Phe	2.41	2.40	−0.01	0.4	0.99	0.95	−0.04	4.0	2.52	2.39	−0.13	5.2
Asp	5.80	5.38	−0.42	7.2	2.04	1.83	−0.21	10.3	5.86	5.59	−0.27	4.6
Glu	8.22	8.28	+0.06	0.7	3.63	3.46	−0.17	4.7	8.37	7.87	−0.50	6.0
Tyr	1.68	1.70	+0.02	1.2	0.69	0.64	−0.05	7.2	2.03	1.88	−0.15	7.4
Lys	2.92	2.91	−0.01	0.3	1.02	0.97	−0.05	4.9	4.62	4.59	−0.03	0.6
His	1.26	1.24	−0.02	1.6	0.54	0.51	−0.03	5.6	1.51	1.38	−0.13	8.6
Arg	3.32	3.16	−0.16	4.8	1.26	1.19	−0.07	5.6	3.77	3.30	−0.47	12.5
Total	41.98	41.55	−0.01	2.5[e]	17.89	17.12	−0.57	4.6[e]	55.12	52.83	−2.29	4.64[e]

[a] Hydrolysis at 145°C, 4 h, sealed ampoule.
[b] All hydrolysates prepared at ABC.
[c] IEC analyses performed at ESCL, single analysis of each hydrolysate.
[d] GC analyses performed at ABC, average of 3 analyses of one hydrolysate.
[e] Average relative % difference.

types were compared to an IEC analysis of the same hydrolysates. To determine which of the amino acids tended to be most subject to GC–IEC variations over the entire sample set, the GC–IEC percent difference for each amino acid was calculated for each sample, and the mean percent differences for all 9 samples were calculated. The percent differences between the GC and IEC values for each amino acid were obtained by dividing the difference between the values by the IEC value, × 100. The first column of Table 1 presents this mean value for 15 amino acids when the same hydrolysate was analyzed by both methods. The differences ranged from 2.1% for alanine and proline to 5.9% for histidine, with an average difference of 3.6%.

Different hydrolysates.—For comparison of GC and IEC analyses of hydrolysates prepared in different laboratories, IEC analyses performed on the hydrolysates prepared at ESCL were compared to GC analyses of the ABC-prepared hydrolysates. This comparison is shown in the second column of Table 1. As expected, when different hydrolysates were analyzed, the mean percent differences for the entire sample set were greater. These percent differences ranged from 2.3% for threonine to 10.1% for valine, with a mean percent difference of 6.3%. The data shown in Table 1 illustrate that over a wide array of sample types the GC and IEC methods provide amino acid values which differ little from each other and that interlaboratory preparation of hydrolysates generally contributes as much or more to differences in amino acid values as does the use of 2 different methods of analysis.

Comparisons of the sums of 15 amino acids from IEC and GC analyses of the hydrolysates are given in Table 2. The data from IEC and GC analyses of the same hydrolysates are very close, with an average percent difference of only 1.9%. Analyses of different hydrolysates by the 2 methods were in good agreement also, with an average percent difference of 5.0%.

The GC and IEC data for 15 amino acids obtained from analyses of the same 9 hydrolysates of different matrixes are given in Tables 3–5, showing the w/w% of each amino acid in each sample, the difference between the IEC and GC values, and the relative percent difference of the values. Tables

Table 4. Comparison of IEC and GC analyses of the same protein hydrolysates[a,b]

Amino acid	Wheat, w/w%				Pruteen, w/w%				NBS Orchard Leaves, w/w%			
	IEC[c]	GC[d]	Diff.	% Rel. diff.	IEC	GC	Diff.	% Rel. diff.	IEC	GC	Diff.	% Rel. diff.
Ala	0.54	0.51	−0.03	5.6	5.27	5.35	+0.08	1.5	0.84	0.85	−0.01	1.1
Val	0.71	0.64	−0.07	9.9	4.23	4.03	−0.20	4.7	0.90	0.86	−0.04	4.4
Gly	0.63	0.60	−0.03	4.8	3.99	3.91	−0.08	2.0	0.78	0.77	−0.01	1.3
Ile	0.55	0.54	−0.01	1.8	3.47	3.56	+0.08	2.3	0.66	0.68	+0.02	3.0
Leu	1.04	1.00	−0.04	3.8	5.45	5.27	−0.17	3.1	1.21	1.24	+0.03	2.5
Pro	1.51	1.48	−0.03	2.0	2.42	2.36	−0.06	2.5	1.24	1.30	+0.06	4.8
Thr	0.40	0.41	−0.01	2.5	3.40	3.35	−0.05	1.5	0.63	0.65	+0.02	3.2
Ser	—	—	—	—	2.33	2.43	+0.10	4.3	—	—	—	—
Phe	0.72	0.70	−0.02	2.8	2.79	2.69	−0.10	3.6	0.79	0.79	0.00	0.0
Asp	0.78	0.71	−0.07	9.0	6.86	6.73	−0.13	1.9	1.63	1.61	−0.02	1.2
Glu	4.33	4.32	−0.01	0.2	7.54	7.30	−0.24	3.2	1.49	1.55	+0.06	4.0
Tyr	0.38	0.38	0.00	0.0	2.25	2.16	−0.09	4.0	0.60	0.53	−0.07	11.6
Lys	0.41	0.39	−0.02	4.9	4.54	4.46	−0.08	1.8	0.72	0.74	+0.02	2.8
His	0.37	0.41	+0.04	10.8	1.38	1.31	−0.07	5.1	0.35	0.38	+0.03	8.6
Arg	0.71	0.66	−0.05	7.0	3.32	3.35	+0.03	0.9	0.73	0.72	−0.01	1.4
Total	13.57	13.42	−0.15	4.7[e]	59.24	58.26	−0.98	2.8[e]	13.18	13.44	+0.08	3.6[e]

[a–e] See Table 3.

Table 5. Comparison of IEC and GC analyses of the same protein hydrolysates[a,b]

Amino acid	NBS Bovine Liver, w/w%				Egg white, w/w%				Ribonuclease, w/w%			
	IEC[c]	GC[d]	Diff.	% Rel. diff.	IEC	GC	Diff.	% Rel. diff.	IEC	GC	Diff.	% Rel. diff.
Ala	3.30	3.43	+0.13	3.9	5.05	5.03	−0.02	0.4	6.90	6.75	−0.15	2.2
Val	3.66	3.63	−0.03	0.8	5.95	5.70	−0.25	4.2	6.92	6.44	−0.48	6.9
Gly	3.15	3.22	+0.07	2.2	3.01	3.03	+0.02	0.7	1.70	1.61	−0.09	5.3
Ile	2.69	2.85	+0.16	5.9	4.33	4.53	+0.20	4.6	2.09	1.86	−0.23	11.0
Leu	5.49	5.44	−0.05	0.9	7.27	7.03	−0.24	3.3	2.03	1.92	−0.11	5.4
Pro	2.78	2.82	+0.04	1.4	3.00	3.06	+0.06	2.0	3.10	3.12	+0.02	0.6
Thr	2.69	2.72	+0.03	1.1	3.78	3.75	−0.03	0.8	7.06	7.13	+0.07	11.0
Ser	2.51	2.70	+0.19	7.6	5.33	5.55	+0.22	4.1	8.31	8.90	+0.57	7.1
Phe	3.28	3.18	−0.10	3.0	5.07	5.05	−0.02	0.4	3.23	3.16	−0.07	2.2
Asp	5.63	5.58	−0.05	0.9	8.87	8.53	−0.34	3.8	13.09	12.36	−0.73	5.6
Glu	7.12	7.30	+0.18	2.5	11.15	10.85	−0.30	2.7	11.70	11.01	−0.69	5.9
Tyr	2.28	2.27	−0.01	0.4	3.35	3.35	−0.00	0.0	6.41	6.42	+0.01	0.2
Lys	—	—	—	—	5.84	5.73	0.11	1.9	9.66	9.33	−0.33	3.4
His	1.52	1.47	−0.05	3.3	2.01	1.90	−0.11	5.5	4.05	3.89	−0.16	4.0
Arg	3.44	3.29	−0.15	4.4	4.84	4.81	−0.03	0.6	4.58	4.77	+0.19	4.1
Total	49.54	49.90	+0.36	2.7[e]	78.85	77.90	−0.95	2.3[e]	90.83	88.67	−2.16	4.4[e]

[a–e] See Table 3.

6–8 present corresponding data in which GC data are from one set of hydrolysates of the 9 matrixes prepared by ABC Laboratories, and IEC data are from a separate set of hydrolysates prepared by ESCL.

Overall, Tables 3–8 confirm that essentially equivalent data are obtained by either analysis method. The sum of the GC amino acid data tends to be slightly less than the corresponding IEC data, although the differences are very small. The GC values for valine were slightly lower than the IEC values, while the GC isoleucine values were generally higher than the corresponding IEC data. Histidine, which has been considered the most difficult amino acid to analyze accurately and reproducibly by GC, showed good GC–IEC agreement across the array of samples. Throughout these analyses, only serine gave more than a single anomalous GC–IEC result. In Tables 3–5, the serine values for soybean meal, poultry feed, wheat, and orchard leaves were 22.1 to 36.7% higher by GC than by IEC (data not shown). The other 5 sample types showed no significant serine GC and IEC differences. In Tables 6–8, the orchard leaves and ribonuclease GC serine values were larger than the IEC values by 20.3 and 19.6%, respectively (data not shown). The serine values were in agreement for the other 7 sample types, and threonine, similar in structure to serine, showed excellent agreement throughout the study.

GC and IEC Analyses of Multiple Hydrolysates

To gain a more comprehensive understanding of the sources and extent of variation in protein hydrolysis and analysis, 3 hydrolysates each of poultry feed and bovine liver were prepared at ESCL using the 145°C, 4 h, screw-cap tube hydrolysis procedure previously described (36). Each hydrolysate was divided, with an aliquot of each of the 6 samples sent to ABC for duplicate analysis by GC; the remainder was retained at ESCL and aliquots were analyzed in duplicate by IEC with the Beckman 121 M analyzer. This study was designed to evaluate reproducibility of hydrolysate preparation and to compare GC and IEC analyses of the same unoxidized hydrolysates.

Tables 9 and 10 present the GC and IEC analyses of the poultry feed and bovine liver hydrolysates. As seen in Table 9, the GC and IEC values from analyses of the same poultry

Table 6. Comparison of IEC and GC analyses of different protein hydrolysates[a]

Amino acid	Soybean meal, w/w%				Poultry feed, w/w%				Fish meal, w/w%			
	IEC[b]	GC[c]	Diff.	% Rel. diff.	IEC	GC	Diff.	% Rel. diff.	IEC	GC	Diff.	% Rel. diff.
Ala	2.08	2.05	−0.03	1.4	1.07	1.08	+0.01	0.9	4.08	3.86	−0.22	5.4
Val	2.56	2.27	−0.18	7.7	1.04	0.97	−0.07	6.7	3.26	2.92	−0.34	10.4
Gly	2.06	2.02	−0.04	1.9	0.92	0.92	0.00	0.0	4.61	4.43	−0.18	3.9
Ile	2.26	2.29	+0.03	1.3	0.86	0.87	+0.01	1.2	2.78	2.64	−0.14	5.0
Leu	3.76	3.59	−0.17	4.5	1.80	1.73	−0.07	3.9	4.72	4.25	−0.47	10.0
Pro	2.48	2.38	−0.10	4.0	1.33	1.27	−0.06	4.5	3.00	2.86	−0.15	4.7
Thr	1.84	1.86	+0.02	1.1	0.71	0.73	+0.02	2.8	2.59	2.54	−0.05	1.9
Ser	2.28	2.43	+0.15	6.6	0.85	0.96	+0.11	12.9	2.22	2.33	+0.11	5.0
Phe	2.46	2.40	−0.06	2.4	1.00	0.95	−0.05	5.0	2.59	2.39	−0.20	7.7
Asp	5.46	5.38	−0.08	1.5	1.98	1.83	−0.15	7.6	6.13	5.59	−0.54	8.8
Glu	8.65	8.28	−0.37	4.3	3.60	3.46	−0.14	3.9	8.68	7.87	−0.81	9.3
Tyr	—	—	—	—	0.59	0.64	+0.05	8.5	2.02	1.88	−0.14	6.9
Lys	3.02	2.91	−0.11	3.6	1.02	0.97	−0.05	4.9	4.90	4.59	0.31	6.3
His	1.25	1.24	−0.01	0.1	0.52	0.51	−0.01	1.9	1.36	1.38	+0.02	1.5
Arg	3.41	3.16	−0.25	7.3	1.24	1.19	−0.05	4.0	3.86	3.30	−0.56	14.5
Total	43.47	42.26	−1.21	3.4[d]	18.53	18.08	−0.48	4.5[d]	56.80	52.83	−3.97	6.8[d]

[a] Hydrolysis at 145°C, 4 h, sealed ampoule.
[b] Analyses at ESCL of hydrolysates prepared at ESCL.
[c] Analyses at ABC of hydrolysates prepared at ABC.
[d] Average relative % difference.

Table 7. Comparison of IEC and GC analyses of different protein hydrolysates[a]

Amino acid	Wheat, w/w%				Pruteen, w/w%				NBS Orchard Leaves, w/w%			
	IEC[b]	GC[c]	Diff.	% Rel. diff.	IEC	GC	Diff.	% Rel. diff.	IEC	GC	Diff.	% Rel. diff.
Ala	0.54	0.51	−0.03	5.6	5.52	5.35	−0.17	3.1	0.93	0.85	−0.08	8.6
Val	0.72	0.64	−0.08	11.1	4.44	4.03	−0.41	9.2	0.98	0.86	−0.12	12.2
Gly	0.62	0.60	−0.02	3.2	4.30	3.91	−0.39	9.1	0.86	0.77	−0.09	10.5
Ile	0.58	0.54	−0.04	6.9	3.66	3.56	−0.10	2.7	0.74	0.68	−0.06	8.1
Leu	1.06	1.00	−0.06	5.7	5.66	5.27	−0.39	6.9	1.34	1.24	−0.10	7.5
Pro	1.52	1.48	−0.04	2.6	2.35	2.36	+0.01	0.4	1.35	1.30	−0.05	3.7
Thr	0.41	0.41	0.00	0.0	3.28	3.35	+0.07	2.1	0.65	0.65	0.00	0.0
Ser	0.60	0.67	+0.07	11.7	2.18	2.43	+0.25	11.5	—	—	—	—
Phe	0.72	0.70	−0.02	2.8	2.81	2.69	−0.12	4.3	0.86	0.79	−0.07	8.1
Asp	0.72	0.71	−0.01	1.4	6.94	6.73	−0.21	3.0	1.74	1.61	−0.13	7.5
Glu	4.58	4.32	−0.26	5.7	7.60	7.30	−0.30	3.9	1.69	1.55	−0.14	8.3
Tyr	0.40	0.38	−0.02	5.0	2.19	2.16	−0.03	1.4	0.46	0.53	+0.07	15.2
Lys	0.42	0.39	−0.03	7.1	4.73	4.46	−0.27	5.7	0.82	0.74	−0.08	9.8
His	0.36	0.41	+0.05	13.9	1.38	1.31	−0.07	5.1	0.38	0.38	0.00	0.0
Arg	0.72	0.66	−0.06	8.3	3.48	3.35	−0.13	3.7	0.75	0.72	−0.03	4.0
Total	13.97	13.42	−0.55	6.1[d]	60.52	58.26	−2.26	4.9[d]	13.55	12.67	−0.88	7.4[d]

[a–d] See Table 6.

feed hydrolysates were very close. The cystine and methionine differences will be discussed in the following section; the GC tyrosine and arginine values are somewhat lower than the IEC values, but histidine, traditionally one of the most difficult to analyze by GC, agreed with the IEC data, showing that the exercise of careful technique allows excellent quantitation of histidine as well as other amino acids. The GC arginine levels were unexpectedly lower than the IEC arginine values in each case, and the cystine values by IEC were erroneously low, because they were obtained from unoxidized hydrolysates. The results of the analyses of the 3 bovine liver hydrolysates are given in Table 10, again showing agreement of GC and IEC values. For these hydrolysates, GC gave a greater lysine value than did IEC, while IEC again gave the larger arginine value. IEC analyses of unoxidized cystine were variable and again erroneously low. The total amino acids found in the hydrolysates were essentially identical by both methods. Because the sets of 3 hydrolysates were prepared at the same time under identical conditions by the same person experienced in hydrolysate preparation, it might be expected that differences between the GC and IEC analyses of the same hydrolysate would be greater than the differences between identically prepared hydrolysates. However, slight differences in the amounts of certain amino acids present in the different hydrolysates can be observed. Thus, variations arise due to the hydrolysis itself, even under preparation conditions most conducive to reproducibility.

GC and IEC Analysis of Cystine and Methionine

As described in the previous presentation by Zumwalt et al. (36), ion-exchange chromatographic values for cystine, and to a much lesser extent methionine, are increased when protein-containing samples are treated with performic acid prior to hydrolysis, and these 2 amino acids are then analyzed as cysteic acid and methionine sulfone. Therefore, we compared the GC analyses of cystine and methionine in unoxidized hydrolysates with the cystine and methionine values obtained by IEC from oxidized and unoxidized hydrolysates.

These data are given in Table 11, with each value representing duplicate analyses of 3 independent hydrolysates. The GC analyses were performed on hydrolysates prepared by ABC, and the IEC analyses were performed on unoxidized and oxidized hydrolysates prepared at ESCL.

The GC cystine values of bovine liver, ribonuclease, and soybean meal (unoxidized) differed from the IEC (oxidized) values by 1.0, 5.2, and 5.5%, respectively. The GC values for methionine, however, were lower than the IEC values for soybean meal and bovine liver, whereas, unexpectedly, the

Table 8. Comparison of IEC and GC analyses of different protein hydrolysates[a]

Amino acid	NBS Bovine Liver, w/w%				Egg white, w/w%				Ribonuclease, w/w%			
	IEC[b]	GC[c]	Diff.	% Rel. diff.	IEC	GC	Diff.	% Rel. diff.	IEC	GC	Diff.	% Rel. diff.
Ala	3.81	3.43	−0.38	10.0	5.60	5.03	−0.57	10.2	6.98	6.75	−0.23	3.3
Val	4.20	3.63	−0.57	13.6	6.68	5.70	−0.98	14.7	6.83	6.44	−0.39	5.7
Gly	3.62	3.22	−0.40	11.0	3.28	3.03	−0.25	7.6	1.68	1.61	−0.07	4.2
Ile	3.11	2.85	−0.26	8.4	4.90	4.53	−0.37	7.6	2.36	1.86	−0.50	21.2
Leu	6.27	5.44	−0.83	13.2	7.96	7.03	−0.93	11.7	1.90	1.92	+0.02	1.1
Pro	3.14	2.82	−0.32	10.2	3.44	3.06	−0.38	11.0	3.26	3.12	−0.14	4.3
Thr	2.82	2.72	−0.10	3.5	3.88	3.75	−0.13	3.4	6.74	7.13	+0.39	5.8
Ser	2.58	2.70	+0.12	4.7	5.36	5.55	+0.19	3.5	7.44	8.90	+1.46	19.6
Phe	3.56	3.18	−0.38	10.7	5.66	5.05	−0.61	10.8	3.32	3.16	−0.16	4.8
Asp	6.14	5.58	−0.56	9.1	9.51	8.53	−0.98	10.3	12.86	12.36	−0.50	3.9
Glu	8.24	7.30	−0.94	11.4	12.27	10.85	−1.42	11.6	11.61	11.01	−0.60	5.2
Tyr	2.52	2.27	−0.25	9.9	3.58	3.35	−0.23	6.4	6.34	6.42	+0.08	1.3
Lys	3.64	3.36	−0.28	7.7	6.42	5.73	−0.69	10.7	9.91	9.33	−0.58	5.9
His	1.76	1.47	−0.29	16.5	2.22	1.90	−0.32	14.4	4.02	3.89	−0.13	3.2
Arg	3.89	3.29	−0.60	15.4	5.24	4.81	−0.43	8.2	4.60	4.77	+0.17	3.7
Total	59.30	53.26	−6.04	10.4[d]	86.00	77.90	−8.1	9.5[d]	89.85	88.67	−1.18	6.2[d]

[a–d] See Table 6.

Table 9. GC and IEC analyses of multiple hydrolysates of poultry feed[a]

Amino acid	Hydrolysate No. 1, w/w% GC[b]	1, w/w% IEC[c]	2, w/w% GC	2, w/w% IEC	3, w/w% GC	3, w/w% IEC
Ala	1.12	1.08	1.12	1.13	1.06	1.07
Val	1.04	1.00	1.07	1.06	0.95	0.95
Gly	0.92	0.93	0.94	0.99	0.87	0.94
Ile	0.86	0.84	0.90	0.89	0.75	0.78
Leu	1.79	1.74	1.84	1.87	1.74	1.78
Pro	1.27	1.26	1.30	1.33	1.25	1.28
Thr	0.71	0.74	0.73	0.77	0.69	0.74
Ser	0.96	0.96	0.97	0.94	0.96	1.00
Met	0.16	0.28	0.14	0.31	0.15	0.32
Hyp	0.03	0.08	0.01	0.09	0.02	0.09
Phe	0.97	0.98	1.00	1.06	0.93	0.99
Asp	1.93	1.91	2.03	2.14	1.86	1.87
Glu	3.73	3.65	3.86	3.85	3.68	3.64
Tyr	0.50	0.60	0.56	0.70	0.53	0.66
Lys	1.00	0.98	1.01	1.06	0.91	0.95
His	0.61	0.55	0.59	0.58	0.51	0.54
Arg	1.18	1.27	1.16	1.37	1.07	1.26
Cys	0.85	0.16	0.48	0.26	0.66	0.22
Total	19.63	19.01	19.71	20.40	18.59	19.08

[a] Hydrolyzed at 145°C, 4 h at ESCL.
[b] Duplicate analyses by ABC, same hydrolysate.
[c] Duplicate analyses by ESCL, same hydrolysate.

Table 10. GC and IEC analyses of multiple hydrolysates of NBS Bovine Liver[a]

Amino acid	Hydrolysate No. 1, w/w% GC[b]	1, w/w% IEC[c]	2, w/w% GC	2, w/w% IEC	3, w/w% GC	3, w/w% IEC
Ala	3.56	3.42	3.83	3.59	3.65	3.52
Val	3.83	3.79	4.09	3.95	3.87	3.84
Gly	3.42	3.27	3.64	3.50	3.51	3.44
Ile	2.93	2.77	3.18	2.86	2.99	2.80
Leu	5.63	5.52	5.80	5.86	5.74	5.80
Pro	2.92	2.85	3.08	2.92	3.02	2.98
Thr	2.74	2.70	2.81	2.78	2.82	2.78
Ser	2.67	2.62	2.76	2.72	2.76	2.75
Met	1.24	1.52	1.29	1.56	1.29	1.55
Hyp	<0.01	0.36	<0.01	0.35	0.14	0.28
Phe	3.28	3.23	3.40	3.38	3.31	3.35
Asp	5.74	5.71	5.88	5.93	5.80	5.84
Glu	7.70	7.61	7.78	7.97	7.69	7.92
Tyr	2.16	2.36	2.38	2.48	2.21	2.47
Lys	4.12	3.10	3.99	3.30	3.94	3.27
His	1.62	1.74	1.60	1.80	1.73	1.78
Arg	3.12	3.64	3.11	3.80	2.56	3.78
Cys	1.09	0.38	0.98	0.66	0.87	0.57
Total	57.77	56.59	59.60	59.41	57.90	58.72

[a] Hydrolyzed at 145°C, 4 h at ESCL.
[b] Duplicate analyses by ABC.
[c] Duplicate analyses by ESCL.

ribonuclease GC value was appreciably greater than the IEC values.

In soybean meal and bovine liver, the GC values for cystine in unoxidized hydrolysates were nearer to the IEC oxidized values than to the IEC unoxidized values. This most likely reflects the less rigorous exclusion of atmospheric oxygen from samples prepared for IEC analysis than for samples prepared for GC analysis.

To evaluate the effects of our hydrolysis procedure on the recovery of cystine, standard solutions of cystine alone were analyzed directly, after hydrolysis and after oxidation and hydrolysis. Samples were prepared at 3 concentrations which represented protein containing 5, 0.5, and 0.2% cystine.

As seen in Table 12 at the 500 μg (5%) level, 68.7% of the cystine remained after hydrolysis, with 10.1% being converted to cysteic acid. The trend toward increased conversion of cystine to cysteic acid was seen at the 50 μg (0.5%) and 20 μg (0.2%) levels. For the samples which were subjected to hydrolysis only, the combined values of cystine plus cysteic acid ranged from 58.6 to 78.8% of the original cystine levels, while after oxidation plus hydrolysis the recovery of cystine as cysteic acid ranged from 95.8 to 97.6%.

This study emphasized to us the importance of the exclusion of oxygen during the preparation of hydrolysates for GC analysis to prevent losses of cystine and methionine. As we (ESCL) routinely oxidize separate samples for cystine and methionine analysis by IEC, we do not as thoroughly remove air from HCl hydrolysates as does ABC, which routinely analyzes amino acid hydrolysates by GC. The data show that more complete removal of oxygen would likely have improved the agreement of the GC and IEC values for methionine in soybean meal and bovine liver.

Recovery of Amino Acids by GC from Orchard Leaves Spiked with Ribonuclease

To gain information on the effects of protein–carbohydrate matrix interactions on the recovery of amino acids from hydrolysis, NBS Orchard Leaves were spiked with ribonuclease prior to hydrolysis at 145°C, 4 h. In this study, 19.1 mg ribonuclease was added to 189.3 mg orchard leaves, and the mixture was hydrolyzed as previously described. Analyses of the hydrolysates were performed by GC as described earlier. Repeated analyses of the orchard leaves and ribonuclease earlier had established the amino acid content of each material, and those data were used to calculate the recovery of each amino acid in the orchard leaves plus ribonuclease hydrolysates. As seen in Table 13, the recoveries of amino acids as determined by GC were very good, indicating no significant destructive interactions of the complex orchard leaves matrix with the ribonuclease protein matrix during hydrolysis.

Experiences of a Commercial Laboratory (Comments by Floyd E. Kaiser)

Experiences during the last 17 years on the GC analysis of amino acids at Analytical BioChemistry Laboratories, Inc., have encompassed some unique and interesting situations not usually encountered in other types of laboratories. These problems centered in 3 areas: (*a*) the wide diversity of sample types submitted by clients; (*b*) lack of information concerning the sample, such as the general composition and matrix; and (*c*) selection of the method and sample preparation. The

Table 11. GC and IEC analyses of cystine and methionine[a]

Matrix	Amino acid	IEC[b] Not oxidized	IEC[b] Oxidized[d]	GC[c] Not oxidized
Soybean meal	Cys	0.53	0.72	0.68
	Met	0.55	0.65	0.41
Bovine liver	Cys	0.54	0.99	0.98
	Met	1.55	1.54	1.27
Ribonuclease	Cys	5.40	5.56	5.27
	Met	3.42	3.44	4.11

[a] Each value is the mean of duplicate analyses of 3 independent hydrolysates, w/w%.
[b] Hydrolyzed and analyzed by ESCL.
[c] Hydrolyzed and analyzed by ABC.
[d] Performic acid oxidized before hydrolysis.

Table 12. Recovery of cystine and cysteic acid after 6N HCl hydrolysis[a]

	Recovery, %			
	After hydrolysis		After oxidation plus hydrolysis	
Cystine, μg[b]	Cystine	Cysteic acid	Cystine	Cysteic acid
500 (5%)	68.7	10.1	0	97.6
50 (0.5%)	16.3	42.3	0	96.4
20 (0.2%)	0	73.2	0	95.8

[a] Hydrolyzed at 145°C, 4 h in 10 mL 6N HCl.
[b] Solutions containing 500, 50, and 20 μg cystine represent protein containing 5, 0.5, and 0.2% cystine, respectively.

sample matrixes which we have analyzed by GC for amino acids on a commercial basis have spanned a wide variety of types, e.g., meat and bone meal, feather meal, hydrolyzed shoe leather, all types of grains and forages, algae, beer, livestock rations, poultry feeds, various types of fish meal, tobacco, pure amino acid mixtures, and simulated lunar samples. Conceivably, the efficiency of analysis of any of these sample types could be improved by studies to define the optimum analytical procedures for a particular sample type. However, a commercial laboratory does not have the luxury of time or expense for research to specially tailor preparation or analysis methods to each sample type received; therefore, the methodology which is most useful is that which is applicable to a broad spectrum of sample types. The methods for amino acid analysis presented here have been found to be excellent methods for the measurement of amino acids on a routine, service basis, in an extremely wide variety of sample types.

A second problem facing the commercial laboratory is the lack of information concerning the sample(s). The nature of the sample, the approximate protein content, prior sample treatment, and the analytical data sought must be made known to the analyst. For example, if a poultry ration is described as "methionine-fortified," was methionine or methionine hydroxy analog used for supplementation? If a sample is described as "high" in one particular amino acid by the person requesting the analysis, it is helpful to know the definition of "high." If the interest is determination of "free" amino acid content, agreement must be reached on the definition of "free." Are the amides of glutamic acid and aspartic acid important to the study? A wealth of information can be provided by an amino acid analysis, but it can be greatly improved by clearly defining the nature of the sample submitted and the objectives sought from the analysis by the client.

Extensive information has been published on the determination of amino acids, whether by ion-exchange chromatography, gas chromatography, or liquid chromatography. The choices of methods are many. Of equal importance, however, is sample preparation. Sample homogeneity, representation, fineness, protein-to-acid ratio during hydrolysis, concentration of acid, temperature and time of hydrolysis, and length of time after hydrolysis until the sample is analyzed are all important to the analysis. An effort should be made to standardize amino acid analysis to some degree or at best provide a minimum of 2 reference materials (animal and vegetable protein) which could be utilized by the analyst to determine the performance of their analysis. The National Bureau of Standards provides several biological standards which can be used for this purpose. In the future, this type of quality assurance control will become available and routine.

Table 13. Recovery of amino acids from a mixture of NBS Orchard Leaves and ribonuclease[a,b]

Amino acid	Calculated, mg	Found, mg	Recovery, %
Ala	2.90	2.89	99.7
Val	2.86	2.85	99.7
Gly	1.77	1.79	101.1
Ile	1.65	1.66	100.6
Leu	2.72	2.77	101.8
Pro	3.06	3.12	102.0
Thr	2.59	2.62	101.2
Ser	3.16	3.22	101.9
Met	0.99	1.05	106.1
Hyp	0.25	0.31	124.0
Phe	2.10	2.11	100.5
Asp	5.41	5.37	99.3
Glu	5.03	5.03	100.0
Tyr	2.23	2.14	96.0
Lys	3.18	3.17	99.7
His	1.46	1.51	103.4
Arg	2.27	2.20	96.9
Cys	1.35	1.35	100.0

[a] Hydrolysis at 145°C, 4 h.
[b] Each value represents 2 independent analyses by GC of 2 separate hydrolysates.

In the thousands of samples we have analyzed, GC of amino acids has provided a reliable method for the determination of amino acids as well as having high versatility, accuracy, and precision.

Summary

Gas chromatographic determinations of amino acids in hydrolysates of 9 diverse sample types (soybean meal, poultry feed, fish meal, wheat grain, Pruteen, NBS Orchard Leaves, NBS Bovine Liver, egg white, and ribonuclease) were compared with ion-exchange chromatographic analyses to gain insight into the effects of the method of analysis on variation in amino acid results, specifically to obtain GC–IEC comparative data on individual amino acids in a range of sample matrixes.

To determine which of the amino acids tended to be most subject to GC–IEC variations over the sample set, the percent difference between the GC and IEC values obtained from the same hydrolysates was calculated. The mean difference for individual amino acids ranged from 2.1% for alanine and proline to 5.9% for histidine, with an average difference of 3.6%. Thus, the GC and IEC values from analyses of the same hydrolysates were very close. To evaluate the level of amino acid variation introduced when 2 different laboratories prepare hydrolysates of the same material, hydrolysates were prepared by 2 laboratories and analyzed by GC and IEC. The mean differences for the entire sample set were greater, ranging from 2.3% for threonine to 10.3% for valine, with an average difference of 6.3%.

The data from the GC and IEC analyses of amino acids in the various matrixes show that essentially equivalent data are obtained from these analytical methods. Histidine, which has generally been considered the most difficult amino acid to analyze accurately and reproducibly by GC, shows good GC–IEC agreement across the array of samples.

To ascertain the extent of amino acid variation introduced by hydrolysate preparation and to relate that factor to GC–IEC analytical variations, multiple hydrolysates of poultry feed and bovine liver were prepared, and aliquots of each hydrolysate were analyzed by GC and IEC. As the sets of 3 hydrolysates of each sample were prepared under identical conditions by the same person experienced in hydrolysate preparation, it could be expected that differences between

the GC and IEC analyses of the same hydrolysate would be greater than the differences between identically prepared hydrolysates. However, slight differences in the amounts of certain amino acids could be observed between hydrolysates of the same material. Thus, the possibility of variations which arise as the result of hydrolysis itself, even under conditions most conducive to reproducibility, should be recognized by the analyst.

In many instances, cystine and methionine are analyzed by IEC as cysteic acid and methionine sulfone after oxidation of the sample and hydrolysis. We therefore compared GC analyses of cystine and methionine in unoxidized hydrolysates with the cystine and methionine values obtained by IEC from oxidized and unoxidized hydrolysates. The GC values for cystine in bovine liver, ribonuclease, and soybean meal differed from the oxidized/IEC values by 1.0, 5.2, and 5.5%, respectively. The GC values for methionine were lower than the IEC values for soybean meal and bovine liver, but, unexpectedly, the GC ribonuclease data were significantly greater than the IEC values.

This study also emphasized to us the importance of the exclusion of oxygen during the preparation of hydrolysates for GC analysis to prevent losses of cystine and methionine. The data show that more complete removal of oxygen would likely have improved the agreement of the GC and IEC values for methionine in soybean meal and bovine liver.

This part of our study was done to evaluate the GC analysis of cystine in hydrolysates, given the difference in ion-exchange chromatographic values for cystine in some matrixes when analyzed directly as cystine and after oxidation to cysteic acid. In this study the GC cystine values compared well with the IEC values after oxidation to cysteic acid, leading to the conclusion that for these matrixes, GC offers a better means for analysis of cystine directly in 6N HCl hydrolysates than does IEC, and is comparable to the performic acid–cysteic acid–IEC technique which requires preparation and analysis of 2 separate hydrolysates.

To determine the effects of matrix-induced destruction of amino acids, we hydrolyzed ribonuclease in the presence of a 10-fold greater amount of orchard leaves. The recoveries were very good, indicating no significant destructive interactions of the complex orchard leaves matrix with ribonuclease during hydrolysis at 145°C, 4 h.

Conclusion

The data demonstrate that GC and IEC analyses of a wide range of sample types agree very well, and that these methods of analysis can be expected to yield essentially identical results when applied to the same hydrolysate. These data also demonstrate that GC–IEC agreement is so close that interlaboratory differences in hydrolysate preparation of the same sample, even when using the same hydrolysis technique, contribute as much to variation in amino acid results as does the method of analysis. Thus, collaborative studies on the amino acid analysis of proteins should recognize that interlaboratory variation in amino acid analyses of proteinaceous material will be due as much to sample handling and hydrolysis technique as to the analysis itself.

Acknowledgments

This research was supported by the Missouri Agricultural Experiment Station, Journal Series No. 10101, and the Missouri Research Assistance Act program.

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Quantitative Analysis of Cystine, Methionine, Lysine, and Nine Other Amino Acids by a Single Oxidation-4 Hour Hydrolysis Method

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The sulfur-containing amino acids cystine and methionine play important roles in animal, especially avian, nutrition. Because these sulfur-containing amino acids are destroyed to varying extents by 6N HCl hydrolysis, oxidation and hydrolysis of cystine to cysteic acid and methionine to methionine sulfone have been widely used for determination of cystine and methionine. Lysine is considered the next limiting amino acid after the sulfur amino acids in poultry nutrition; therefore, determination of the amino acid content of rations focuses first on these 3 amino acids. The objective of this investigation was to establish whether lysine and other amino acids could be accurately determined in proteinaceous materials which had undergone performic acid oxidation. To perform this evaluation, lysine was determined in a variety of protein-containing materials both with and without performic acid oxidation. Performic acid oxidation followed by 6N HCl hydrolysis at 145°C for 4 h allows accurate measurement of 3 amino acids especially important to poultry nutrition, cystine, methionine, and lysine, in a single preoxidized hydrolysate; this method can be extended to another 9 protein amino acids.

The sulfur-containing amino acids cystine and methionine play important roles in animal, especially avian, nutrition. Because these sulfur-containing amino acids are destroyed to varying extents by 6N HCl hydrolysis, oxidation and hydrolysis of cystine to cysteic acid and methionine to methionine sulfone have been widely used for determination of cystine and methionine (1–7). Accurate assessment of the essential amino acid (excluding tryptophan) content of poultry rations by ion-exchange chromatography (IEC) has, therefore, required the preparation and analysis of 2 hydrolysates of each ration to be assayed. One portion of a ration sample undergoes performic acid oxidation before 6N HCl hydrolysis, followed by IEC analysis, for cystine and methionine; another portion of the same sample is directly hydrolyzed with 6N HCl for determination of the other amino acids.

MacDonald et al. (8) reported a collaborative study by 7 laboratories of the determination of cystine and methionine in food and feed ingredients using performic acid oxidation and 6N HCl hydrolysis. The matrixes studied were isolated soy protein, soybean meal, corn meal, wheat middlings, wheat flour, nonfat dry milk, and a purified protein, β-lactoglobulin. The samples were oxidized for 16 h with performic acid in 125 mL boiling flasks, and HBr was used to destroy the excess performic acid. The samples were then evaporated to dryness, 6N HCl was added, and the flasks were purged with nitrogen and stoppered and then placed in a 100°C oven for 18 h for hydrolysis. Following hydrolysis, the hydrolysates were analyzed for cysteic acid and methionine sulfone by ion-exchange chromatography. For the determination of cystine as cysteic acid, the coefficients of variation between laboratories for duplicate samples of the 6 ingredients ranged from 7.13 to 10.8%, and the corresponding values for methionine as methionine sulfone ranged from 1.18 to 12.8%. Recoveries of cystine and methionine were determined by analysis of β-lactoglobulin and comparison with sequence data. The mean recovery of cystine was 95% with a range of 91–101%, and the methionine mean recovery was 101% with a range of 98–106%. Analyses of other amino acids after oxidation and hydrolysis were not reported.

Investigators at the National Institute of Animal Science and the Royal Veterinary and Agricultural University, Copenhagen, performed extensive studies aimed at the development of a "streamlined" hydrolysate preparation method for determining the amino acid composition of feedstuffs (9). Their principal conclusion was that all the common acid-stable feed amino acids except tyrosine and perhaps histidine can be accurately measured in hydrolysates of oxidized samples when phenol is included in the oxidation reagent.

The method was then subjected to a collaborative trial within the European Economic Community (9). Hydrolysates were prepared and analyzed by the various participating laboratories, and aliquots were returned by each laboratory to the coordinating laboratory (Copenhagen) for additional analyses. About 27% of the data submitted by 25 laboratories were rejected for various reasons, and 26% of the data from coordinating laboratory analyses of the submitted hydrolysates were rejected. In both cases, the total rejection of data from 4 laboratories accounted for 15–16% of available results. For the hydrolysates analyzed by the Copenhagen laboratory, values for cystine, phenylalanine, isoleucine, valine, serine, and threonine were excluded most often, reflecting the sensitivity of these amino acids to oxidation or hydrolysis conditions. The amino acids values excluded most often from the data provided by the collaborating laboratories were those for methionine, proline, cystine, serine, and aspartic acid. The highest mean coefficients of variation were observed for cystine, arginine, histidine, methionine, isoleucine, phenylalanine, serine, and valine.

Elkin and Griffith (10) studied the effect of oxidative pretreatment on the analysis of amino acids in sorghum grains by using a modification of the Copenhagen procedure. Comparing oxidized and nonoxidized samples, they found that oxidative pretreatment resulted in increased values for cystine and methionine and destroyed tyrosine and phenylalanine and concluded that the complete (except tryptophan) amino acid content of a feedstuff cannot be determined using the modified Copenhagen method.

Lysine is considered the next limiting amino acid after the sulfur amino acids in poultry nutrition; therefore, determination of the amino acid content of rations focuses first on cystine, methionine, and lysine. The efficiency with which rations can be assayed would be greatly increased if these 3 amino acids, plus perhaps other amino acids, could be quan-

Accepted June 9, 1986.
Presented at the Symposium on Chromatography of Amino Acids, AOAC 99th Annual International Meeting, Oct. 27–31, 1985, Washington, DC.

titatively determined in a single hydrolysate with a single chromatographic analysis.

As background information, attention is called to a paper by Gehrke et al. (11) on amino acid analysis which discusses sample preparation for chromatography of amino acids and acid hydrolysis of proteins. Included is the use of a glass tube with Teflon-lined screw cap as the hydrolysis vessel, high-temperature, short-time hydrolysis, performic acid oxidation of cystine and methionine, multiple hydrolysis times at 145°C, and interlaboratory preparation of hydrolysates. A diverse sample set was used in the study, which included a range of protein-containing matrixes. Ion-exchange chromatography was used for the amino acid analyses.

The objective of this investigation on the measurement of amino acids was to establish whether lysine and other amino acids could be accurately determined in proteinaceous materials which had undergone performic acid oxidation. To perform this evaluation, lysine was determined in a variety of protein-containing materials both with and without performic acid oxidation.

Experimental

Apparatus

(a) *Amino acid analyzer.*—Beckman (Palo Alto, CA) 121 M amino acid analyzer.

(b) *Hydrolysis and evaporation.*—Aluminum heat block unit with holes to accept 25 mm od screw-cap culture tubes was used for sample hydrolysis.

Hydrolysis vessels were 25 × 150 mm glass culture tubes with Teflon-lined screw caps (Corning No. 9826).

For removal of air from sample and hydrolysis vessel, one-hole rubber stopper which fits mouth of glass tube was fitted with glass "T." One arm of "T" was attached to vacuum line (water aspirator), the other to nitrogen gas supply (with valves to control nitrogen flow).

Evaporation equipment consisted of an aluminum block with holes to accept 20 mL conical glass cups and a Plexiglas manifold fitted to top of block. Manifold provided openings to direct stream of N_2 into each sample cup, and 2 aspirators connected to ports on block provided evacuation, while N_2 flow was controlled by flowmeter.

Reagents

High-purity, 6N HCl (GFS Chemical, Columbus, OH, Cat. No. 504) was used. Performic acid was prepared by adding 1.0 mL 30% H_2O_2 to 9.0 mL 88% formic acid. The solution was allowed to stand at room temperature for 1 h, then cooled to 0°C in ice bath before use.

Chromatographic Conditions

(a) *Nonoxidized hydrolysates.*—Column 2.8 × 690 mm. Resin: Benson B-X8, particle size 8 ± 1 μm, bed height 320 mm. Buffer flow rate 10.0 mL/h; ninhydrin 5.0 mL/h. Buffers: (*1*) pH 2.85, 0.20N Na^+ ion, 0.20N citrate ion, 5% isopropanol; (*2*) pH 3.10, 0.16N Na^+ ion, 0.20N citrate ion; (*3*) pH 3.51, 0.20N Na^+ ion, 0.20N citrate ion; (*4*) pH 3.90, 0.40N Na^+ ion, 0.20N citrate ion; (*5*) pH 7.13, 1.00N Na^+ ion, 0.20N citrate ion. Sample diluting buffer pH 2.20, 0.20N Na^+ ion, 0.20N citrate ion. Sodium hydroxide 0.20N NaOH with 1% Na_2EDTA. Buffer change times: 24.4, 35.5, 53.2, and 67.7 min. Column temperatures 55 and 68°C; change time 72.8 min. Analysis time through arginine 126 min; total time between injections 128.5 min. Recorder 1.9 absorbance unit, full scale; chart speed 6 in./h. Detector wavelengths: 570 nm, alpha amino acids; 440 nm, Pro, Hydroxy Pro; 690 nm, reference. Flow cell pathlength 12 mm. Typical amino acid concentrations 0.125 μmole/mL each. Volume of sample injected 50 μL.

Buffers (*1*)–(*5*) were preserved with 4 drops/L of a pentachlorophenol solution (5 mg/L in 95% ethanol). Buffers (*1*)–(*4*) contained 10 mL/L 30% solution of thiodiglycol solution to preserve methionine. Sample buffer contained 20 mL/L thiodiglycol solution and 1 mL/L 90% phenol as a preservative. Buffers were prepared with high-purity, 6N HCl, deionized water (Barnstead Nanopure) and were refrigerated. All elution buffers were filtered through 0.2 μm filters (Millipore).

(b) *Oxidized hydrolysates.*—Buffers: (*1*) pH 2.85, 0.20N Na^+ ion, 0.20N citrate ion, 5% isopropanol; (*2*) pH 7.13, 1.00N Na^+ ion, 0.20N citrate ion (used 4 min/run to aid column regeneration). Sodium hydroxide 0.20N NaOH, 1% Na_2EDTA. Column temperature 55°C. Analysis time through serine 46.5 min; total time between injections 52.4 min.

Sample buffer was the same as for nonoxidized hydrolysates, except thiodiglycol was not required. Elution buffers were identical to buffers (*1*) and (*5*) in nonoxidized hydrolysate chromatographic conditions.

Sample Preparation

(a) *Unoxidized samples.*—Sample containing ca 20 mg protein was weighed exactly into 25 × 150 mm glass culture tubes; 10 mL of 6N HCl was added to sample. (*Note*: Alternatively, 10.0 mL of 6N HCl containing norleucine [e.g., 328 μg/mL] was used for hydrolysis and was redissolved in pH 2.2 buffer without norleucine in a later step. The addition of norleucine before hydrolysis eliminates need to use exact volumes of sample or buffer in succeeding steps.) Rubber stopper with glass "T" (attached to vacuum and nitrogen lines) was fitted into mouth of tube. With no nitrogen flow, pressure in tube was reduced to ca 20 mm Hg, and the tube was held in an ultrasonic bath for ca 10 s to remove dissolved air from solution. Tube was purged with nitrogen (low flow), then pressure reduction, sonication, and nitrogen purge steps were repeated 3 to 5 times.

The flow of nitrogen was slowly increased to produce slight pressure in tube, rubber stopper was removed carefully, and the tube was quickly closed with Teflon-lined screw cap and tightened firmly. Tube was placed in 145°C ± 2° block heater for 4 h, then removed from block and allowed to cool to room temperature. Hydrolysate was quantitatively transferred to 100 mL volumetric flask with deionized water, made to volume, mixed, and allowed to stand for ca 1 h, or a portion was filtered through glass fiber filter in a Gooch crucible.

An aliquot (e.g., 3.0 mL) of the hydrolysate was transferred to an evaporation cup and evaporated to dryness in block evaporator at 65°C and 260 mm Hg pressure. Residue was redissolved (with sonication) in aliquot (e.g., 3.0 mL) of Na citrate buffer at pH 2.2 containing norleucine (e.g., 32.8 μg/mL) and thiodiglycol. (See *Note* above.)

Approximately 1.2 mL of sample was transferred to 1.5 mL polypropylene centrifuge tube and centrifuged at 13 000 × *g* for 3 min. A 50 μL aliquot of supernatant was analyzed by automated ion-exchange amino acid analyzer. Sample was stored at −20°C if not analyzed immediately.

(b) *Oxidized samples.*—Sample containing ca 10 mg protein was weighed exactly into 25 × 80 mm glass hydrolysis tube with Teflon-lined screw cap. (Tubes were made from 25 × 150 mm glass culture tubes by the University of Missouri–Columbia glassblowing service.) Five mL cold performic acid was added to sample and maintained in an ice

Table 1. Recovery of lysine following oxidation and hydrolysis (animal origin)

Sample type	Lysine, % (w/w)[a] Hydrolysis only	Oxidation + hydrolysis	% Recovery after oxidation
Blood meal	6.420	6.314	98.3
Fish concentrate	4.918	4.897	99.6
Poultry meal	3.090	3.054	98.8
Meat and bone meal	2.528	2.660	105.2
Fish	3.433	3.616	105.3
Animal protein	2.382	2.370	99.5
Animal by-products	3.223	3.220	99.9
Casein	7.339	7.312	98.8
Poultry concentrate	2.218	2.309	104.1
	2.212	2.174	98.3
Mean			100.8
Std. dev.			2.89

[a] Values represent single or multiple assays.

bath in a refrigerator or cold room overnight. For readily soluble proteins, reaction time of 4 h at 0°C is sufficient.

After oxidation, 1 mL 48% HBr was added (in hood) while tube was swirled in ice bath. After initial reaction had subsided, sample tubes were removed from ice bath and allowed to warm to room temperature.

Tubes were placed for 1 h in *unheated* block evaporator with tube holder, and pressure was reduced to ca 380 mm Hg. Each tube was purged with stream of nitrogen gas throughout evaporation. Pressure was decreased to 260 mm Hg (by reducing N flow), block temperature was set at 65°C, and samples were evaporated to dryness (8–12 h). This procedure prevents foaming and loss of sample.

Five mL 6N HCl was added to dried samples; tubes were tightly capped with Teflon-lined caps and hydrolyzed at 145°C for 4 h.

After samples were allowed to cool, HCl was removed by evaporation in block evaporator for ca 8–12 h. Reduced pressure of 260 mm Hg was applied from beginning of evaporation; temperature was 65°C. The sample was then redissolved (with sonication) in 20.0 mL aliquot pH 2.2 buffer (thiodiglycol not needed).

Approximately 1.2 mL sample was transferred to 1.5 mL polypropylene centrifuge tube and centrifuged at 13 000 × *g* for 3 min. A 50 μL aliquot of supernate was analyzed by automated ion-exchange amino acid analyzer for cysteic acid and methionine sulfone. Sample was stored at −20°C if not analyzed immediately.

Table 2. Recovery of lysine following oxidation and hydrolysis (plant origin)

Sample type	Lysine, % (w/w)[a] Hydrolysis only	Oxidation + hydrolysis	% Recovery after oxidation
Soybean meal	3.077	3.160	102.7
	2.846	2.853	100.2
Sunflower meal	1.075	1.052	97.9
Cottonseed meal	1.962	1.809	92.4
Corn gluten meal	1.217	1.174	96.5
Corn	0.249	0.243	97.6
	0.328	0.326	99.4
Cereal fines	0.263	0.259	98.5
Leaves	1.059	1.054	99.5
Corn leaves	0.643	0.645	100.3
Mean			98.5
Std. dev.			2.75

[a] Values represent single or multiple assays.

Table 3. Recovery of lysine following oxidation and hydrolysis (poultry rations)

Sample type	Lysine, % (w/w)[a] Hydrolysis only	Oxidation + hydrolysis	% Recovery after oxidation
Fryer starter	1.203	1.220	101.4
Poultry ration	1.121	1.170	104.4
Poultry feed	0.682	0.691	101.3
Mixed ration	1.261	1.212	96.1
Poultry feed	1.207	1.164	96.4
Poultry ration	1.58	1.50	94.9
	1.11	1.06	96.4
	1.08	1.06	98.1
	1.13	1.08	95.6
	1.07	1.05	98.1
Mean			98.3
Std. dev.			3.11

[a] Values represent single or multiple assays.

Results

Lysine values in hydrolysates of materials of animal origin, with and without performic acid oxidation, are shown in Table 1. Comparisons of the "oxidation plus hydrolysis" values with the "hydrolysis only" data show that performic acid oxidation provides quantitative recovery of lysine, and thus permits an accurate measurement of lysine along with cystine and methionine in a single step oxidation-hydrolysis method.

A similar comparison was made with proteinaceous substances of plant origin, and the results are shown in Table 2. The recovery of lysine after performic acid oxidation again was quantitative, with the only anomalous result that for cottonseed meal, with a recovery of 92.4%.

As cystine, methionine, and lysine are of particular interest in poultry feed, Table 3 shows the recovery of lysine after performic acid oxidation of a number of feeds. Again, the data show that lysine is not susceptible to losses during oxidation and can be accurately measured along with cysteic acid and methionine sulfone after performic acid oxidation of the matrix followed by hydrolysis with 6N HCl at 145°C for 4 h.

The recovery of lysine from these matrixes is summarized in Table 4, showing the overall lysine recovery from 30 different samples to be 99.2%. Two pure proteins, β-lactoglobulin A from bovine milk and ribonuclease A from bovine pancreas, were also compared. Lysine recoveries of 99.3 and 99.2%, respectively, were obtained after oxidation and hydrolysis.

The quantitative determination of lysine following oxidation and 6N HCl hydrolysis of a broad range of sample types led to the evaluation of the recovery of other amino acids following oxidation. Eighteen samples of various types and matrixes were compared, and the findings were: (*1*) Ten additional amino acids gave recoveries between 90 and 110%

Table 4. Recovery of lysine followng oxidation and hydrolysis (summary)[a]

Samples	Average[b] recovery, %	SD
Animal origin feeds	100.8	2.89
Plant origin feeds	98.5	2.75
Rations 16 to 25% crude protein	98.3	3.11
Overall (30 samples)	99.2	2.92

[a] Miscellaneous samples not included.
[b] Ten samples, each type.

after oxidation and hydrolysis as compared to hydrolysis alone. These amino acids were: aspartic and glutamic acids, glycine, threonine, proline, alanine, valine, leucine, isoleucine, and arginine from animal origin. (*2*) Tyrosine and histidine gave recoveries below 90%. (*3*) Serine and phenylalanine gave inconsistent results; serine had recoveries of less than 90% for 13 samples, from 90 to 100% for 4 samples, and over 100% for one sample. Phenylalanine values were also inconsistent, with indications that an unknown ninhydrin-positive component coeluted with phenylalanine in the oxidized samples, yielding in several instances recoveries above 100%.

Discussion

We have shown that performic acid oxidation followed by 6N HCl hydrolysis at 145°C for 4 h allows accurate measurement of 3 amino acids especially important to poultry nutrition, cystine, methionine, and lysine, in a single preoxidized hydrolysate, and that this method can be extended to another 9 protein amino acids. We have designated this the CML + 9 method.

The initial objective of this investigation was to develop a more rapid yet highly accurate means of assessing the cystine, methionine, and lysine content of feed and feed ingredients, because such an improvement would allow more timely and efficient utilization of protein sources by the agricultural sector. We found that one preoxidized hydrolysate prepared at 145°C for 4 h will indeed provide these data.

The single hydrolysis method represents an opportunity to achieve considerable labor savings with retention of high accuracy. Analytical accuracy is achieved as evidenced by high recoveries of amino acids from well characterized proteins. Thus, the amino acid values of feed ingredients and formulated poultry rations obtained by the CML + 9 method can be relied upon as values which best represent the diet fed the animal. In this manner, a stronger data base for each species of animal can be developed.

Application of the CML + 9 method to feed ingredients has been extensively presented throughout this paper. If poultry rations supplemented with the DL-methionine analog, DL-2-hydroxy-4-(methylthio)butanoic acid, are analyzed by this method, one must take into account the supplement in addition to the amino acid found.

Methionine, the first limiting amino acid for broilers and turkey poults, must be added to poultry rations to achieve maximum growth and feed conversions. Commercial DL-2-hydroxy-4-(methylthio)butanoic acid is added to poultry rations as a liquid feed supplement to help meet the sulfur amino acid requirements of the bird. Thus, an analytical value for the analog should be obtained by an independent analysis of each ration assayed by the CML + 9 method. In this manner, one avoids any numerical confusion from the final total methionine values. Rapid and accurate analytical methods for DL-2-hydroxy-4-(methylthio)butanoic acid have been reported using a variety of instrumental methods of measurement for either compound (12–14; personal communication, R. R. Flynn and W. D. Shermer, Monsanto Chemical Co., 1985).

It is interesting to note that DL-2-hydroxy-4-(methylthio)butanoic acid will not be detected in the CML + 9 method because the 2-hydroxy group does not derivatize, whereas the 2-amino group of methionine does. One can add the supplemental 2-hydroxy-4-(methylthio)butanoic acid value to the natural methionine value obtained by the CML + 9 method and obtain the total methionine value for the poultry ration.

It is important to cite these different approaches to avoid compiling analytical data in growth studies which are obtained by several different analytical methods. Such compilation could possibly bias interpretation of the growth and feed conversion results within each study. By citing the differences, the analyst shows diligence and reports the most accurate amino acid values to the nutritionist.

In summary, there is a distinct need in the private sector to develop, define, and institute rapid methods for specific assays of cystine, methionine, and lysine in poultry feeds and feed ingredients. This need can currently best be met by the use of sample oxidation (in the case of the sulfur amino acids), then a rapid hydrolysis, followed by analysis designed to meet the specific needs.

Acknowledgment

This research was supported by the Missouri Agricultural Experiment Station and the Missouri Research Assistance Act program, Journal Series No. 10057. The authors thank Betty Rudel for her technical assistance during this investigation.

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TECHNICAL COMMUNICATIONS

Determination of Adulterated Natural Bitter Almond Oil by Carbon Isotopes

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Bitter almond oil (benzaldehyde), a flavoring compound used in many foods, was isolated from apricot kernels; 2 synthetic benzaldehyde samples were obtained from commercial sources. All samples were analyzed for radiocarbon (^{14}C) content. The natural sample yielded a value consistent with its natural origin (approximately 116% of Modern Standard Activity), while the synthetic samples were devoid of ^{14}C activity as expected for a petrochemical material. Implications for quality control of bitter almond oil are discussed.

Bitter almond oil is an important flavoring found in many foods; it is the basis for natural cherry flavoring. Because natural bitter almond oil is approximately 95% benzaldehyde, commercial benzaldehyde is often used as an artificial substitute. Foods are often formulated to taste like cherry by the addition of "natural" and/or "artificial" benzaldehyde, with a corresponding claim on the product label. Artificial benzaldehyde is manufactured from inexpensive fossil fuel sources, while the natural flavor is expensive and in limited supply; the price differential between natural and artificial benzaldehyde is nearly 50-fold as of this writing. With the current consumer trend toward natural foods, there is an obvious economic advantage to adulterate natural flavors with artificial chemicals and fraudulently certify them as natural.

Analysis for the natural radiocarbon content of foodstuffs has been used as a tool to distinguish natural products and synthetics from those from fossil fuel sources. Atmospheric CO_2 and natural products derived from it via plant photosynthesis contain minute quantities of the radioactive isotope ^{14}C. Because ^{14}C has a radioactive half-life of approximately 6000 years, carbon which has been in place for millions of years is essentially devoid of ^{14}C, it having long since decayed away. ^{14}C analysis can be used as a sensitive indicator of the source of carbon.

The use of ^{14}C analysis in the food industry was first suggested for ethanol in 1952 (1). It has since been applied to fermented spirits (2–4), vinegar (5), caffeine (6), cinnamon (7), citric acid (8), and other flavor chemicals (9–11). We will demonstrate the applicability of ^{14}C analysis as a quality control tool for natural bitter almond oil.

Experimental

Samples

Apricot kernels (0.5 kg) were milled to a greasy paste in a Wiley mill. The paste was suspended in water (2 L) for 24 h. The suspension was steam-distilled in an efficient hood (*Caution:* HCN is generated!) and the distillate (1 L) was extracted twice with 50 mL portions of ether. The ether layers were combined and dried over $MgSO_4$, and the solvent was evaporated under reduced pressure. The resulting oil (~3 g) was used for ^{14}C analysis. Commercial benzaldehyde was obtained from commercial sources.

^{14}C Analysis

An approximately 2 g sample was placed in a 2 L combustion bomb, the bomb was charged with 100 psi O_2, and the sample was ignited electrically. When the pressure surge subsided and the bomb had cooled, the resultant gases were passed through traps of dry ice/trichloroethylene and liquid O_2. (*Caution:* Observe proper precautions in the handling of liquid O_2, which is used instead of liquid N_2 to avoid O_2 condensation in the gas line.) The liquid O_2 trap was isolated, and any residual noncondensible gas was evacuated. An aliquot of the CO_2 was taken for ^{13}C analysis (12). The remaining CO_2 was converted to methane with tritium-free H_2 over 0.5% ruthenium on alumina at 475°C (13). The methane was purified by passing through a trap of dry ice–trichloroethylene, trapping on silica gel at liquid N_2 temperature, and evacuating excess H_2. Methane was released by warming the silica gel trap and freezing the evolved methane in a liquid N_2 trap. It was then expanded into a storage flask to await ^{14}C counting. The ^{14}C activity of methane was determined by counting for 1000 min in a low level proportional gas counter with anti-coincidence circuitry. The counter was calibrated using the NBS Oxalic Acid Reference Standard, and counter background was measured using CO_2 from a 300-million-year-old marble with no ^{14}C activity. The $\delta^{13}C$ data were used to correct ^{14}C activities for isotope fractionation (5).

Discussion

The results of our ^{14}C analyses show that natural bitter almond oil isolated in our laboratory yielded a "modern" value (see Table 1), i.e., a ^{14}C activity value of approximately 120% of Modern Standard Activity as expected in recently fixed carbon sources (see Table 2). The samples of commercial benzaldehyde had no significant ^{14}C activity. As with other materials studied, the samples of benzaldehyde from natural and synthetic sources are very different in their ^{14}C content.

The determination of ^{14}C in natural bitter almond oil thus constitutes a sensitive means of detecting adulteration of bitter almond oil with synthetic benzaldehyde from petro-

Table 1. ^{14}C analysis of natural bitter almond oil and benzaldehyde

Sample	$\delta^{13}C$	^{14}C, % MSA[a]
Bitter almond oil	−28.5	116
Benzaldehyde No. 1	−27.0	<1
Benzaldehyde No. 2	−26.6	2

[a] 100% Modern Standard Activity is 95% of the 1950 activity of NBS Oxalic Acid. 100% MSA equals a specific ^{14}C activity of 13.56 dpm/g carbon.

Table 2. Known ^{14}C (5, 14, 15) of atmospheric CO_2[a]

Year	% MSA	Year	% MSA
1975	139	1980	130
1976	136	1981	127
1977	134	1982	125
1978	133	1983	123
1979	131	1984	120

[a] The standard deviation of recent atmospheric ^{14}C measurements is ca 2% MSA for any given year.

Received November 6, 1985. Accepted April 3, 1986.

chemical sources. The large difference between natural and synthetic values should allow for detection of small quantities (approximately 10–15%) of added synthetic material. Proper interpretation of the results must take into account the minor year-to-year changes in atmospheric ^{14}C levels (see Table 2). The analysis of a large number of proprietary samples in our laboratories has revealed that undeclared substitution of petrochemical benzaldehyde for natural benzaldehyde is an important problem in the flavor industry.

While this method is effective in determining addition of petrochemically derived benzaldehyde, it is not expected to be effective in determining benzaldehyde synthesized from agricultural feedstocks. This is implied by Bricout and Koziet (9), who found that citral from turpentine is indistinguishable radiometrically from citral isolated from lemon oil.

Acknowledgment

Isotopic measurements were performed by Krueger Enterprises, Inc., Geochron Laboratories Division, Cambridge, MA.

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Determination of Residual Chlorinated Solvents in Decaffeinated Coffee by Using Purge and Trap Procedure

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A purge and trap method developed for the analysis of volatile halocarbons (VHCs) in table-ready foods has been applied to the determination of residual chlorinated solvents in decaffeinated coffee. Samples are stirred in water and purged with nitrogen for 0.5 h in a water bath at 100°C. The analytes are collected on a duplex trap composed of Tenax TA and XAD-4 resin, eluted with hexane, and determined by gas chromatography (GC) with a thick-film, wide-bore capillary column and Hall electrolytic conductivity detector. The higher levels of residual chlorinated solvents are also confirmed by full scan GC/mass spectrometry. Samples are analyzed for several chlorinated solvents including methylene chloride, chloroform, 1,2-dichloroethane, methyl chloroform, carbon tetrachloride, trichloroethylene, and tetrachloroethylene. Eleven decaffeinated instant coffees and 14 decaffeinated ground coffees representing 9 different commercial brands were analyzed by this method. Residues of methylene chloride, chloroform, methyl chloroform, and trichloroethylene were found. Highest levels occurred in ground coffees—up to 640 ppb methylene chloride. Recoveries of these 4 chlorinated solvents detected from fortified samples ranged from 96 to 106%. Brews from 3 instant and 3 ground coffee samples contained up to 1.1 ppb and 4.8 ppb methylene chloride, respectively. In addition, the resulting grounds from the brewing process were analyzed and contained up to 14 ppb methylene chloride and up to 13 ppb trichloroethylene. The limit of quantitation for these coffee grounds was 4 ppb for methylene chloride. Chromatograms from this purge and trap method are clean, enabling levels of about 10 ppb and 0.4 ppb to be achieved for chlorinated solvents in decaffeinated coffees and their brews, respectively.

Received February 18, 1986. Accepted June 4, 1986.

Reference to any commercial material, equipment, or process does not, in any way, constitute endorsement or recommendation by the Food and Drug Administration.

Chlorinated solvents have long been used in the production of decaffeinated instant and ground roasted coffees (1, 2). Trichloroethylene, once the chief chlorinated solvent used in the decaffeination process, has been indicted as toxic and has fallen from use (3, 4). Currently, methylene chloride is the only chlorinated solvent used in the United States and Canada. However, recent information has indicated that methylene chloride may present a risk of human cancer from certain exposures (5, 6). Consequently, the U.S. Food and Drug Administration has established an allowable level of 10 ppm methylene chloride in decaffeinated ground roasted and decaffeinated instant coffees (7).

One of the first attempts to analyze decaffeinated coffee for residual chlorinated solvents involved gas phase pyrolysis with a total chloride determination (8). Somewhat later, a chloride-specific membrane electrode was used for the direct potentiometric determination of chloride ions after the chlorinated solvents were sparged from decaffeinated coffee in boiling water. The limit of detection for methylene chloride was 1 ppm (9). Other investigators have used azeotropic distillation to isolate the residual chlorinated solvents followed by gas chromatographic (GC) determination with flame ionization (FID) (10, 11) or electron capture detection (ECD) (12, 13). Brandenberger et al. used ethyl acetate extractions with both GC/ECD and GC with microcoulometric detection (14). More recently, Page and Charbonneau (15) determined methylene chloride and trichloroethylene by both GC/ECD and GC with Coulson electrolytic conductivity detection (GC/CECD). A closed system vacuum distillation technique with toluene as the carrier solvent was used to isolate the residual

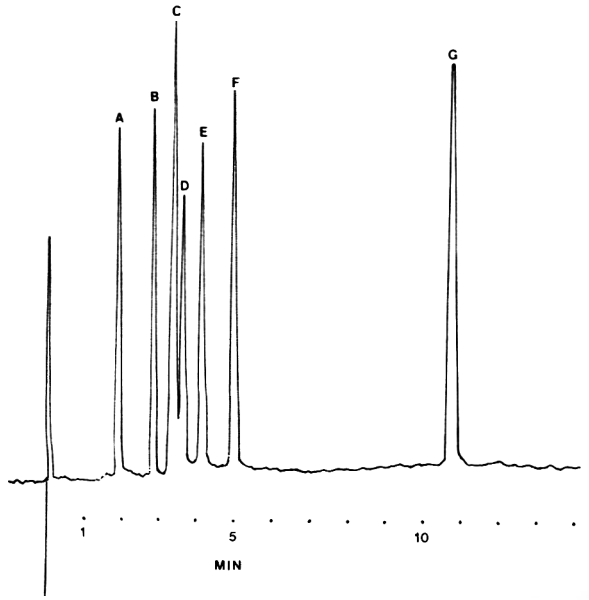

Figure 1. Chromatogram of chlorinated solvent standards with Hall electrolytic conductivity detection (GC/HECD); methyl silicone (5 μm film) fused silica capillary column, 50 m × 0.53 mm, 65°C: A, methylene chloride (650 pg); B, chloroform (650 pg); C, 1,2-dichloroethane (1100 pg); D, methyl chloroform (600 pg); E, carbon tetrachloride (660 pg); F, trichloroethylene (730 pg); G, tetrachloroethylene (1000 pg).

Table 1. Results of analysis of instant decaffeinated coffee for residual chlorinated solvents[a]

Sample	Analyte[b] Methylene chloride	Chloroform	Methyl chloroform	Trichloroethylene
Brand A				
Lot 1	18	0.0	0.0	0.0
Lot 2	22, 21	9.9, 11	0.0, 0.0	0.0, 0.0
Brand B				
Lot 1	11, 13	10, 13	0.0, 0.0	0.0, 0.0
Lot 2	21	10	0.0	0.0
Brand C				
Lot 1	17, 17	24, 24	0.0, 0.0	0.0, 0.0
Brand D				
Lot 1	32	10	0.0	0.0, 0.0
Brand E				
Lot 1	0.0, 0.0	0.0, 0.0	0.0, 0.0	0.0, 0.0
Lot 2	18	Tr[c]	0.0	0.0
Brand F				
Lot 1	21	Tr	0.0	0.0
Lot 2	44, 49	12, 13	0.0, 0.0	0.0, 0.0
Brand G				
Lot 1	43, 46	Tr, Tr	0.0, 0.0	0.0, 0.0

[a] Values expressed as ppb.
[b] No evidence of any other chlorinated solvents found.
[c] Tr = >2 ppb and <10 ppb.

chlorinated solvents. Levels as low as 0.01 ppm were reported. A headspace isolation technique with glass capillary column GC and FID detection was used by Van Rillaer et al. (16) to determine several chlorinated residual solvents from decaffeination of coffee. The limit of quantitation for this system was approximately 0.5 ppm for trichloroethylene. A second headspace procedure (17) has been applied to the determination of methylene chloride in decaffeinated tea as well as decaffeinated coffee. Packed column GC/CECD analysis allowed quantitation limits of 0.05 ppm.

A purge and trap method which was originally developed for the determination of ethylene dibromide (EDB) in table-ready foods from the Total Diet Program of the U.S. Food and Drug Administration (FDA) (18) was subsequently adapted to the determination of EDB in grains and intermediate grain-based foods (19). Samples were vigorously stirred in water and sparged with a stream of nitrogen while in a boiling water bath. The EDB collected on a Tenax TA trap tube was eluted with hexane and determined by GC/ECD and GC with Hall electrolytic conductivity detection (HECD). A quantitation limit of 1 ppb was obtained. Later, using a collection trap composed of Tenax TA plus Amberlite XAD-4 resin, this purge and trap procedure was used for the multiresidue determination of fumigants in whole grains and intermediate grain-based foods (20). Table-ready foods were also analyzed for 8 volatile halocarbons and carbon disulfide by this modified procedure (21). Through the use of wide-bore capillary columns, limits of quantitation were reduced to less than 1 ppb for halocarbons, using GC/ECD. Methylene chloride determined with HECD had a quantitation limit of 4 ppb.

This study, undertaken in an effort to determine methylene chloride and other chlorinated solvents in instant decaffeinated and ground roasted decaffeinated coffee, used the modified purge and trap procedure described above (20). Eleven decaffeinated instant coffees and 14 decaffeinated ground coffees representing 9 different commercial brands were analyzed.

This modified purge and trap procedure provides a versatility not experienced with headspace techniques, in that the need for a series of calibration measurements for each matrix is eliminated. Sensitivity or limit of quantitation also exceeds that of published headspace methods for the determination of chlorinated solvents in decaffeinated coffees.

METHOD

Principle

Samples in water are extracted (sparged) with a stream of nitrogen with vigorous stirring in a water bath at 100°C. The residual volatile solvents collected on a duplex adsorbent trap composed of Tenax TA and XAD-4 resin and eluted with hexane are determined by gas chromatography with Hall electrolytic conductivity detection (GC/HECD). Higher levels of residues in instant and ground coffee are confirmed by GC/mass spectrometry (GC/MS). Thick-film, wide-bore capillary columns were used in the quantitation of chlorinated solvent residues.

Apparatus and Reagents

The procedures for the preparation of standard stock solutions, working standard solutions, and spiking solutions as well as all other apparatus and reagents are described elsewhere (20).

Determination

The determination is described elsewhere (20).

Results and Discussion

The development of certain specific procedural parameters has been discussed elsewhere (18, 20).

The analysis for chlorinated solvents at low part per billion or sub-part per billion levels requires stringently clean reagents. Procedural blanks must be analyzed frequently to ensure the validity of results. Some solvents (notably methylene chloride and chloroform) are nearly ubiquitous in some

Table 2. Results of analysis of ground decaffeinated coffee for residual chlorinated solvents[a]

Sample	Analyte[b] Methylene chloride	Chloroform	Methyl chloroform	Trichloro-ethylene
Brand A				
Lot 1[d]	15	Tr[c]	Tr	0.0
Lot 2[d]	38	Tr	0.0	0.0
Brand B				
Lot 1[d]	26, 28	Tr, Tr	0.0, 0.0	0.0, 0.0
Lot 2[d]	27, 30	Tr, Tr	0.0, 0.0	0.0, 0.0
Brand C				
Lot 1[e]	42, 43	Tr, Tr	0.0, 0.0	41, 44
Lot 2[e]	230	Tr	Tr	Tr
Lot 3[e]	230, 230	Tr, Tr	28, 31	0.0, 0.0
Brand D				
Lot 1[d]	11, 11	0.0, 0.0	0.0, 0.0	0.0, 0.0
Brand H				
Lot 1[e]	130, 150	0.0, 0.0	0.0, 0.0	0.0, 0.0
Lot 2[e]	150, 160	0.0, 0.0	10, 11	0.0, 0.0
Lot 3[e]	220, 240	27, 28	64, 66	0.0, 0.0
Brand I				
Lot 1[f]	230, 240	25, 25	0.0, 0.0	0.0, 0.0
Lot 2[f]	260, 260	32, 33	Tr, Tr	0.0, 0.0
Lot 3[f]	620, 640	Tr, Tr	0.0, 0.0	0.0, 0.0

[a] Expressed as ppb.
[b] No evidence of any other chlorinated solvents found.
[c] Tr = >2 ppb and <10 ppb.
[d] Made for automatic drip coffee makers.
[e] Made for percolator coffee makers.
[f] Made for all coffee makers.

laboratory environments or as impurities in various reagents. The preparation of these reagents is, therefore, necessarily rigorous to reduce procedural blanks to a negligible level. Other precautions, such as washing glassware before use, and keeping clean solvents and adsorbents in tightly closed containers, are also necessary. This burden, however, is lessened considerably if somewhat higher quantitation levels (e.g., 10 ppb) are acceptable.

Although a limit of quantitation (LOQ) of 4 ppb can be achieved for methylene chloride using this purge and trap procedure, a level of 10 ppb was determined to be adequate for this study. Consequently, the GC/HECD attenuation was adjusted to obtain ½ full scale deflection (½ FSD) on a 1 mV recorder for approximately 800 pg methylene chloride. A sample weight of 10 g was used. An eluate of 3 mL was obtained and the injection volume was 5 μL. Thus, a residue

Table 3. Recoveries from decaffeinated coffee fortified with chlorinated solvents[a]

Sample	Analyte Methylene chloride	Chloroform	Methyl chloroform	Trichloro-ethylene
Instant coffee	99 (38)	102 (80)	102 (78)	99 (76)
	106 (78)	105 (130)	106 (350)	100 (420)
	103 (190)	102 (160)		
	103 (510)	100 (500)		
	98 (1200)	102 (1200)		
Ground coffee	105 (140)	99 (80)	105 (78)	103 (76)
	101 (140)	100 (80)	99 (350)	101 (420)
	102 (160)	101 (100)		
	96 (510)	103 (500)		
	98 (1200)	102 (1200)		

[a] Values expressed as percent recovery (and ppb fortified).

Table 4. Results of analysis of brewed decaffeinated instant coffee for residual chlorinated solvents[a]

Sample	Analyte[b] Methylene chloride	Chloroform	Methyl chloroform	Trichloro-ethylene
Brand D				
Lot 1	0.70, 0.82	0.63, 0.69	0.0, 0.0	0.0, 0.0
Brand F				
Lot 2	1.0, 1.1	0.52, 0.58	0.0, 0.0	0.0, 0.0
Brand G				
Lot 1	0.48, 0.55	0.28, 0.33	0.0, 0.0	0.0, 0.0

[a] Values expressed as ppb; brewed at rate of 2.4 g/250 mL, approximating 1 rounded teaspoon per 6 oz cup.
[b] No evidence of any other chlorinated solvents found.

level of 10 ppb will be represented by approximately 10% FSD. Essentially the same LOQ (10 ppb) was obtained for chloroform, methyl chloroform, and trichloroethylene, using GC/HECD. A chromatogram (GC/HECD) of 7 chlorinated solvents is presented as Figure 1.

The eluates generated from the analysis of decaffeinated instant and decaffeinated, ground, roasted coffees were not determined by GC/ECD. The large number of extraneous peaks precluded reliable quantitation.

Coffee samples used for this survey were obtained from local commercial sources. Seven brands of decaffeinated instant coffee and 6 brands of decaffeinated, ground, roasted coffee were sampled. A total of 25 lots representing 9 different brands were examined. Sample weights were taken from freshly opened glass or metal containers and most determinations were performed in duplicate. Tables 1 and 2 contain the results of these analyses. The highest level of chlorinated solvents in instant coffees was 49 ppb methylene chloride. Ground, roasted coffees contained up to 640 ppb methylene chloride. These values are substantially lower than those reported by Page and Charbonneau (17). In their study, conducted in 1982, 6 coffee samples with North American origin labels were examined by a headspace GC technique; 4 were found to contain levels greater than 800 ppb. The highest level reported was 2130 ppb methylene chloride.

Because of this seeming disparity, several coffee samples were subjected to exhaustive sparging to ensure that all the residual chlorinated solvents were being extracted. After the initial sparging period of 30 min, the collection trap was

Table 5. Results of analysis of brew and grounds from decaffeinated ground coffee for residual chlorinated solvents[a]

Sample	Analyte[b] Methylene chloride	Chloroform	Methyl chloroform	Trichloro-ethylene
Brand C				
Lot 1	0.60, 0.65 (7.2, 7.6)	Tr, Tr (3.3, 3.6)	0.0, 0.0 (0.0, 0.0)	Tr, Tr[c] (13, 13)
Brand H				
Lot 3	2.4, 2.8 (8.4, 8.7)	Tr, Tr (8.6, 8.6)	Tr, Tr (6.5, 6.8)	0.0, 0.0 (0.0, 0.0)
Brand I				
Lot 3	4.4, 4.8 (12, 14)	0.0, 0.0 (6.7, 7.2)	0.0, 0.0 (0.0, 0.0)	0.0, 0.0 (0.0, 0.0)

[a] Values expressed as ppb chlorinated solvents in brew (and grounds); brewed with automatic drip coffee maker at rate of 24 g/L, approximating 1 tablespoon per 6 oz cup.
[b] No evidence of any other chlorinated solvents found.
[c] Tr = <0.4 ppb.

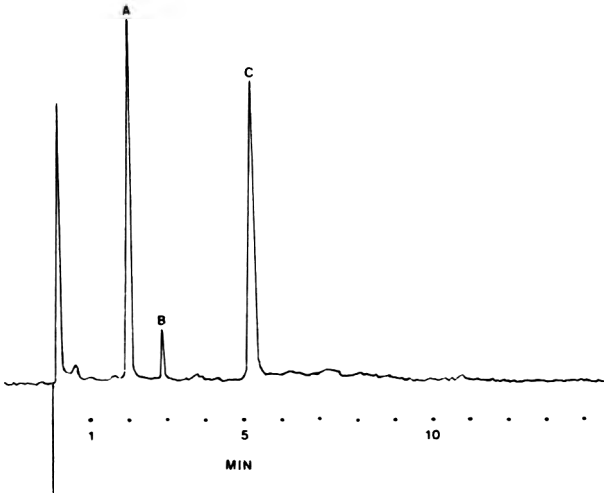

Figure 2. Chromatogram of decaffeinated ground coffee (brand C, lot 1) (equivalent to 16 mg) with Hall electrolytic conductivity detection (GC/HECD); methyl silicone (5 μm film) fused silica capillary column, 50 m × 0.53 mm, 65°C: A, methylene chloride (43 ppb); B, chloroform (trace, <10 ppb); C, trichloroethylene (44 ppb).

replaced with a fresh trap. A second 30 min sparging period produced no additional chlorinated solvent, indicating that all of the chlorinated solvents are extracted in the first sparging period.

As illustrated in Table 3, acceptable recoveries were obtained from coffee samples fortified with up to 1200 ppb methylene chloride. Fortification with chloroform, methyl chloroform, and trichloroethylene also yielded satisfactory recovery. All fortification standards were aqueous solutions. Recoveries for all 4 analytes ranged from 96 to 106%. It can thus be stated with assurance that this purge and trap procedure produces accurate results for the determination of chlorinated solvent residues in instant and ground decaffeinated coffees.

It is suggested that perhaps recent, more efficient manufacturing techniques for the removal of chlorinated decaffeination solvents may be responsible for the variations between the study by Page and Charbonneau and the effort reported here.

Both instant and ground coffees were brewed according to the recipes suggested by the manufacturers. Thus, 250 mL water at approximately 90°C was added to 2.4 g instant coffee,

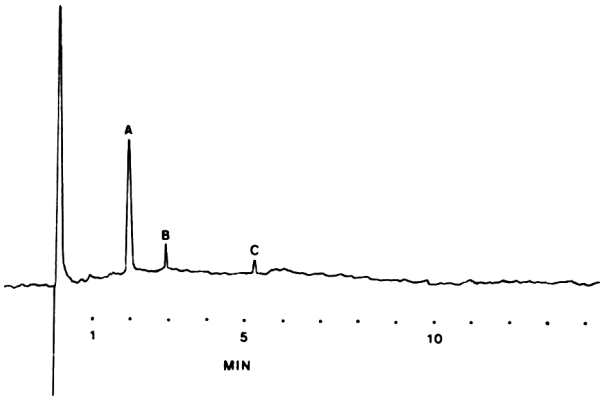

Figure 3. Chromatogram of brewed decaffeinated ground coffee (brand C, lot 1) (equivalent to 400 mg) with Hall electrolytic conductivity detection (GC/HECD); methyl silicone (5 μm film) fused silica capillary column, 50 m × 0.25 mm, 65°C: A, methylene chloride (0.65 ppb); B, chloroform (trace, <0.4 ppb); C, trichloroethylene (trace, <0.4 ppb).

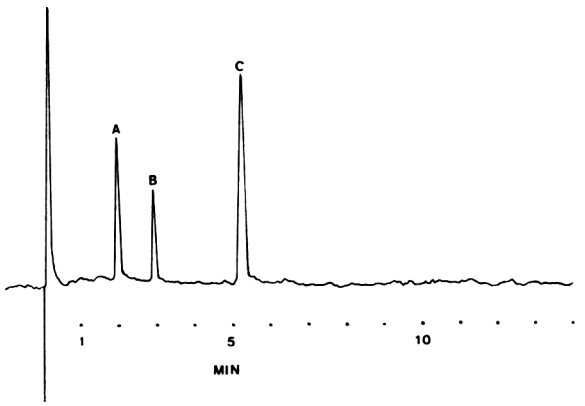

Figure 4. Chromatogram of grounds from decaffeinated ground coffee (brand C, lot 1) (equivalent to 38 mg) with Hall electrolytic conductivity detection (GC/HECD); methyl silicone (5 μm film) fused silica capillary column, 50 m × 0.53 mm, 65°C: A, methylene chloride (7.2 ppb); B, chloroform (3.6 ppb); C, trichloroethylene (13 ppb).

i.e., approximately 1 rounded teaspoon per 6 oz cup. The brew was stirred and allowed to stand 3 min before analysis. The entire 250 mL solution was analyzed as 1 sample weight. Table 4 contains the results of analysis of brews from decaffeinated instant coffee. Likewise, 24 g ground coffee plus 1 L water was brewed with an automatic drip coffee maker (approximately 1 tablespoon per 6 oz cup). A 250 mL aliquot was then analyzed by this purge and trap procedure.

Although 2 ground coffees had shown significant levels of methyl chloroform and trichloroethylene, only traces of these residues were present in the brews. In an effort to account for this loss, the grounds resulting from brewing were analyzed. The entire 24 g of coffee grounds was purged and trapped. The results of analysis of brews and grounds from decaffeinated ground coffees are listed in Table 5 and indicate that these 2 solvents tend to remain in the grounds. It is suggested that methyl chloroform and trichloroethylene tend to remain in the coffee grounds because of their relative insolubility in water. The approximate solubilities of methyl chloroform, trichloroethylene, and methylene chloride in water are 0.7, 1.1, and 20 μL/mL, respectively. This same effect is seen for chloroform, which has a solubility in water of about 5 μL/mL. Figures 2–4 represent GC/HECD chromatograms of eluates from a ground coffee sample, and the brew and grounds of this same sample, respectively. Because of larger sample sizes the LOQs for methylene chloride in brews and coffee grounds are about 0.4 and 4 ppb, respectively.

In conclusion, this purge and trap method is suitable for rapid analysis of residual chlorinated solvents in instant and ground decaffeinated coffees. It shows acceptable recovery from fortified samples and good reproducibility. Results can be obtained in less than 2 h. The procedure is not labor-intensive, in that extractions proceed unattended. Elution of the dual adsorbent trap results in very clean, concentrated eluates suitable for both low level GC/HECD quantitation and GC/MS confirmational analysis.

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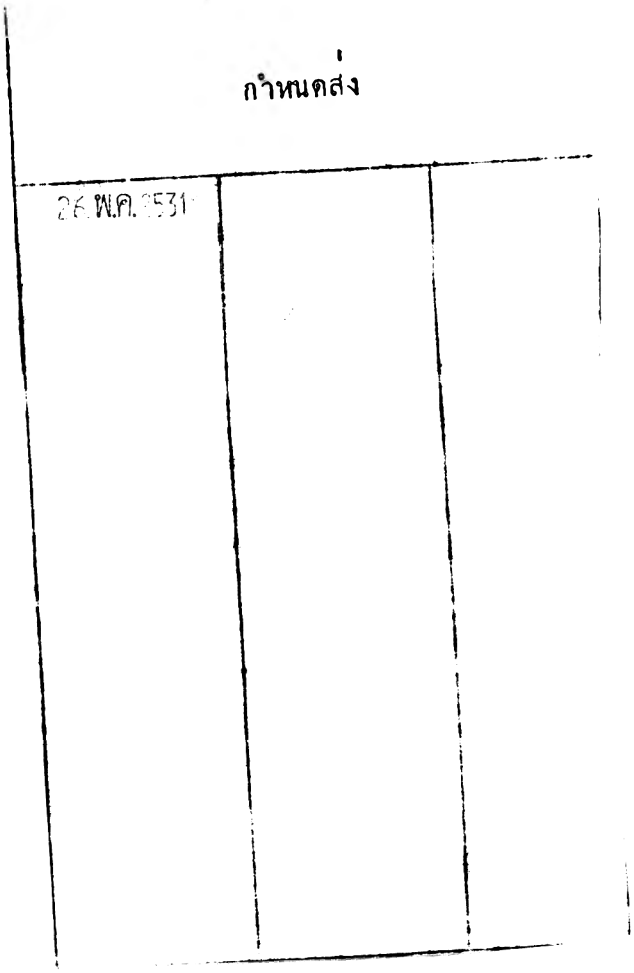

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