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MAY/JUNE 1986
VOL. 69, NO. 3

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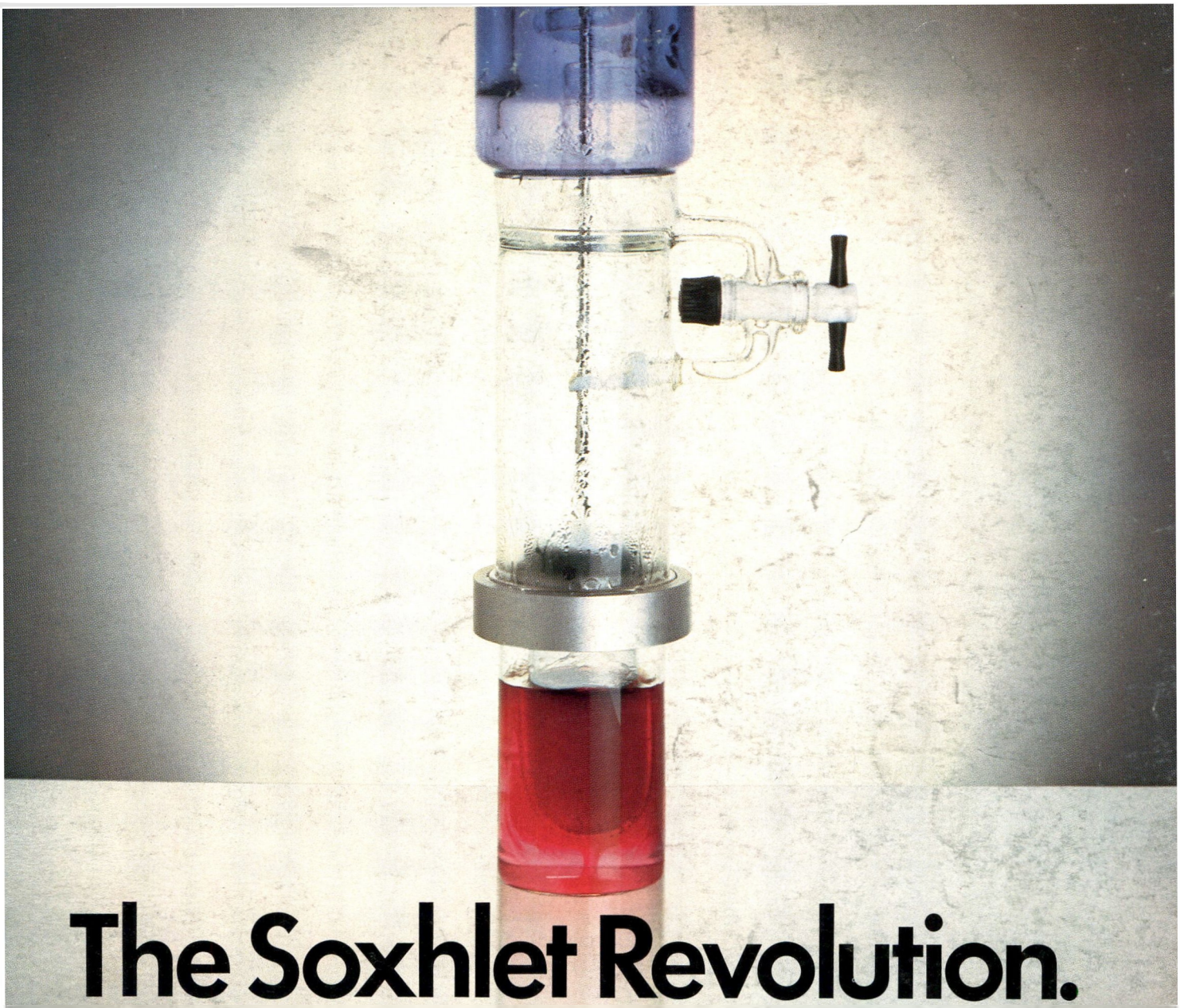

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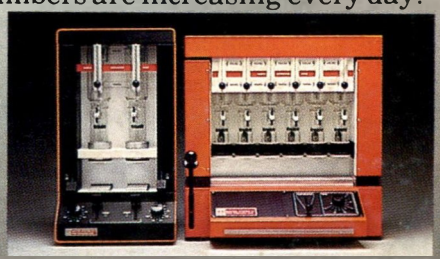

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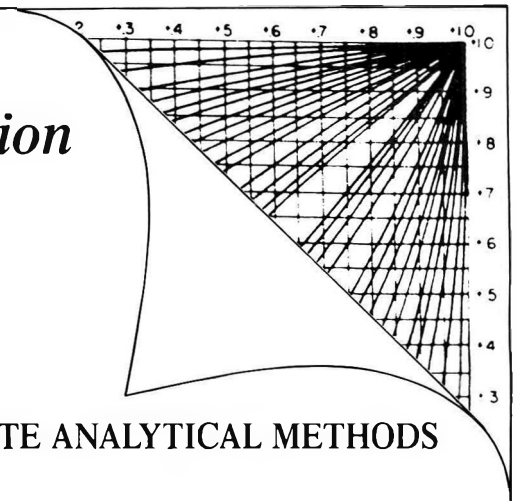

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Grant T. Wernimont, Author
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It is a natural extension of and a valuable addition to the classic Youden-Steiner, *Statistical Manual of the AOAC*.

Contents

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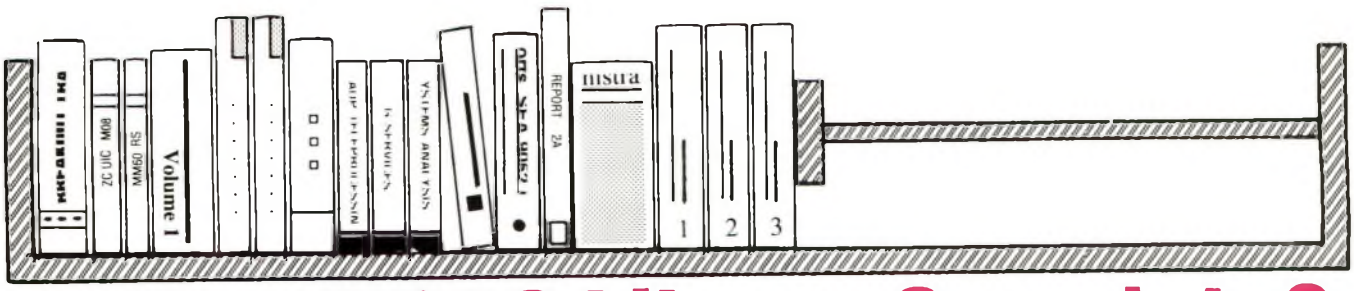

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Basic & Applied Research in the Analytical Sciences Related to Agriculture and the Public Health

MAY/JUNE 1986
VOL. 69, NO. 3

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A New Handbook on How to Set Up or Improve Laboratory Quality Assurance Programs

Quality Assurance Principles for Analytical Laboratories

by Frederick M. Garfield
former Assistant Administrator
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This handbook provides essential information needed to design, document, implement, or improve a laboratory quality assurance program . . . A program that can enable you to document the credibility of your laboratory's analytical data.

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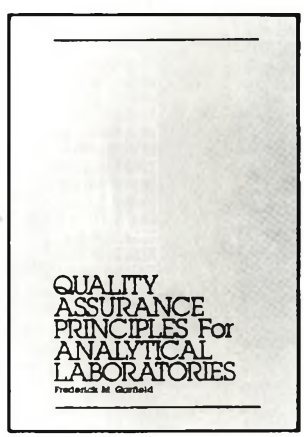

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LITERATURE

Chromatography Newsletter

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pH/ISE Meters Catalog

This 20-page catalog features Orion's entire range of 19 pH and ISE meters, including new high quality, portable meters and new advanced bench-top meters. Specifications, a selection chart, and complete ordering information are included. Contact: Mark Zimmerman, Orion Research Inc., 840 Memorial Dr, Cambridge, MA 02139; 800/225-1480.

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Unz & Co., a leading supplier of hazardous materials, labels, placards, forms, and educational materials to meet the customer's regulatory requirements of DOT, EPA, OSHA, IATA, and NFPA, announces the availability of its 1986 compliance guide. For a free copy of this compliance guide, call 800/631-3098 (in NJ 201/795-5400) or write Mat Ratra, Unz & Co., 190 Baldwin Ave, PO Box 308, Jersey City, NJ 07303.

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The *Journal of Analytical Atomic Spectrometry (JAAS)* is a new bimonthly journal published by the Royal Society of Chemistry, which contains original research papers, short papers, communications, and letters concerned with the development and application of atomic spectrometric techniques. *JAAS* contains comprehensive reviews on specific topics, general information, and news of interest to analytical atomic spectroscopists, including information on forthcoming conferences and book reviews. Special issues of *JAAS* will be published devoted to subjects highlighted by particular symposia. Also included in *JAAS* will be the literature reviews previously covered in *Annual Reports on Analytical Atomic Spectroscopy*. Subscription price: (1986) $319.00/£165.00. Contact: The Royal Society of Chemistry, 30 Russell Square, London WC1B 5DT, England.

ISO Standards Published

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

ISO 6735-1985 Dried milk—Assessment of heat class—Heat-number reference method—$16.00

ISO 6638/1-1985 Fruit and vegetable products—Determination of formic acid content—Part I: Gravimetric method—$12.00

ISO 5492/6-1985 Sensory analysis—Vocabulary—Part 6—$14.00

ISO 6740-1985 Dried whey—Determination of nitrate and nitrite contents—Method by cadmium reduction and spectrometry—$14.00

ISO 7218-1985 Microbiology—General guidance for microbiological examinations—$22.00

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

Meetings

June 16–18, 1986: AOAC Midwest Regional Section Meeting, Lincoln, NE. Contact: Thomas Jensen, Nebraska Department of Agriculture, 3703 S 14th St, Lincoln, NE 68502, USA; 402/471-2176.

June 24–25, 1986: AOAC Northeast Regional Section Meeting, Canisius College, Buffalo, NY. Contact: Gerald A. Roach, Food and Drug Administration, 599 Delaware Ave, Buffalo, NY 14202, USA; 716/846-4494.

August 17–22, 1986: 3rd Biannual Symposium on Diffuse Reflection, Wilson College, Chambersburg, PA. Contact: F. E. Barton, II, Russell Research Center, PO Box 5677, Athens, GA 30613, USA.

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

September 28–October 3, 1986: 13th Annual Federation of Analytical Chemistry and Spectroscopy Societies Meeting (FACSS XIII), Cervantes Convention Center, St. Louis, MO. Contact: Alexander Scheeline, Program Chair, University of Illinois, School of Chemical Sciences, 79 Roger Adams Laboratory Box 48, Urbana, IL 61801, USA; 217/398-1952.

October 5–10, 1986: American Oil Chemists' Society (AOCS) 2nd World Conference on Detergents: Looking Towards the 1990's, Montreux Convention Center, Montreux, Switzerland. Contact: Meetings Manager, AOCS, 508 S 6th St, Champaign, IL 61820, USA.

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The Registry, Scottsdale, Arizona
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October 20–22, 1986: 6th International Symposium on HPLC of Proteins, Peptides, and Polynucleotides (ISPPP), Baden-Baden, West Germany. Those who wish to present a paper must submit an abstract by June 15, 1986. For further information, contact Secretariat, 6th ISPPP, PO Box 3980, D-6500 Mainz, West Germany; (06131) 392284, Telex: 4187476 uni d.

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, USA; 703/522-3032.

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, USA; 703/522-3032.

Short Courses

The State University of New York at Albany will offer 2 short courses this summer—(1) X-Ray Spectrometry, June 2–6 and June 9–13, 1986 and (2) X-Ray Powder Diffraction, June 16–20 and June 23–27, 1986. The first week of both courses will cover basic principles, techniques, and practical applications, and the second week will continue with further fundamentals and practical applications. Registration may be made for the one-week (either week) or two-week sessions. Contact: Henry Chessin: State University of New York at Albany, Department of Physics, 1400 Washington Ave, Albany, NY 12222; 518/442-4513.

American Oil Chemists' Society (AOCS) and POS Pilot Plant Corporation will offer a short course on Processing of Oilseeds/Fats and Oils, July 13–18, 1986, POS Pilot Plant, Saskatoon, Saskatchewan, Canada. Contact: Meetings Manager, AOCS, 508 S 6th St, Champaign, IL 61820.

Massachusetts Institute of Technology will offer a one-week elementary course in Design and Analysis of Scientific Experiments, July 14–19, 1986. Contact: Director of Summer Session, Room E19-356, Massachusetts Institute of Technology, Cambridge, MA 02139.

AOAC Publishes New Statistical Manual

Use of Statistics to Develop and Evaluate Analytical Methods, written by Grant T. Wernimont and edited by William Spendley, is an indispensible reference source for those dealing with collaborative studies. It is a sequel to the world-renowned *Statistical Manual of the AOAC* by W. J. Youden and E. H. Steiner.

The book's first two sections establish the basic concepts of collaborative studies; while these sections should not be taken for granted, it is the latter two sections in which Wernimont and Spendley offer considerably more information on interlaboratory and intralaboratory experimentation than Youden and Steiner and emphasize the importance of this activity in the detection of correctable "bugs" before submission of the method under study to interlaboratory collaboration.

The specific contents include 4 sections:

(1) Introduction—AOAC collaborative studies, organization and procedures for collaborative study, selection of methods for studies, types of interlaboratory study, need for this manual;

(2) The Measurement Process—what is measurement? measurement as a relationship between priorities, measurement as a production process, performance characteristics of a measurement process, developing, evaluating, and using analytical processes, AOAC methods of analysis;

(3) Intralaboratory Development of an Analytical Process—the need for intralaboratory experiments, some requisites for sound experimentation, statistical methodology, experimental plans;

(4) Interlaboratory Evaluation of a Laboratory Process—interlaboratory experiments, objectives for an interlaboratory study, the concept of variance components, planning an interlaboratory study, experiments to compare laboratory performance, evaluating interlaboratory data and formulating precision statements, reporting the results from an interlaboratory study.

In addition, the book contains an index and three appendixes which include tables, statistical computations, and a glossary.

This book can be obtained from AOAC, 1111 N 19th St, Suite 210, Arlington, VA 22209; 703/522-3032. Member price: $47.55 (U.S.)/$50.55 (outside U.S.); Nonmember price: $52.50 (U.S.)/$55.50 (outside U.S.).

New Private Sustaining Member

AOAC welcomes a new private sustaining member to the growing list of firms aware of the need to support an independent methods validation association: Mettler Instrument Corp., Hightstown, NJ.

Correction

The recommendations of Associate Referee D. R. Petrus (*J. Assoc. Off. Anal. Chem.* **68,** 1202(1985)) were not approved by the General Referee and Committee on Foods II. They were, however, approved by the Official Methods Board and adopted by the Association.

Use of Statistics to Develop and Evaluate Analytical Methods. By G. T. Wernimont. Edited by W. Spendley. Published by AOAC, 1111 N 19th St, Suite 210, Arlington, VA 22209, 1985. xvi + 183 pp. Member price: $47.55 (U.S.)/$50.55 (outside U.S.); Nonmember price: $52.50 (U.S.)/$55.50 (outside U.S.). ISBN 0-935584-31-5.

This manual is a sequel to the world-renowned *Statistical Manual of the AOAC* by W. J. Youden and E. H. Steiner. The book comprises four sections. The first two sections establish the basic concepts of collaborative studies; while these sections should not be taken for granted, it is the latter two sections in which Wernimont and Spendley offer considerably more information on interlaboratory and intralaboratory experimentation than Youden and Steiner. Fifty-four tables illustrate statistical analysis of real-life collaborative study data. In addition, the book contains an index and three appendixes which include reference tables, statistical computations, and a glossary.

Solid State Nuclear Magnetic Resonance of Solid Fuels. By D. E. Axelson. Published by Brookfield Publishing Co., Inc., Old Post Rd, Brookfield, VT 05036, 1985. 320 pp. Price: $56.00. ISBN 0-919868-25-8.

This book provides a concise, comprehensive overview of the major theoretical and practical problems encountered in performing carbon-13 solid state nuclear magnetic resonance (NMR) research with emphasis on examples taken from fossil fuel research, and it also provides an overview of the state-of-the-art of application of C-13 NMR techniques to solid fossil fuel analysis and processing.

McGraw-Hill Dictionary of Chemical Terms. Published by McGraw-Hill Book Co., 1221 Avenue of the Americas, New York, NY 10020, 1985. 470 pp. Price: $15.95 (paperback). ISBN 0-07-045417-5.

This dictionary contains more than 6800 chemical terms, with full coverage devoted to the following fields: analytical chemistry, atomic physics, general chemistry, nuclear physics, organic chemistry, physical chemistry, and spectroscopy.

CRC Handbook of Natural Pesticides: Methods. By H. B. Mandava. Published by CRC Press, Inc., 2000 Corporate Blvd, NW, Boca Raton, FL 33431, 1985. Vol. I: 552 pp. Price: $108.00(U.S.)/$125.00(outside U.S.) ISBN 0-8493-3651-1; Vol. II: 568 pp. Price: $112.00(U.S.)/$130.00(outside U.S.) ISBN 0-8493-3652-X.

Naturally occurring pesticides derived from plants, insects, and microorganisms are comprehensively examined. These handbooks provide the most current information available to researchers of the subject in chemistry, biochemistry, physiology, pathology, entomology, microbiology, and to others interested in the development and use of safe pesticides. Volume I covers theory, practice, and detection, while volume II will cover isolation and identification methods.

Food Microbiology and Hygiene. By P. R. Hayes. Published by Elsevier Science Publishing Co., Inc., 52 Vanderbilt Ave, New York, NY 10017, (in North America) and Elsevier Science Publishers, PO Box 211, Amsterdam, The Netherlands (outside North America), 1985. 403 pp. Price:$72.00 ISBN 0-85334-355-1.

Chapters included in this work are Fundamental Principles of Microbiology; Food Poisoning and Other Foodborne Hazards; Food Spoilage; Microbiological Examining Methods; Factory Design and Construction; Factory Layout; Design of Food Processing Equipment; Quality Assurance and Production Control; Cleaning and Disinfection: Practical Application; Waste Disposal; Hygiene and Training of Personnel; Legislation.

CIPAC Handbook 1C: Analysis of Technical and Formulated Pesticides. Compiled by J. Henriet, A. Martijn, and H. H. Povlsen. Published by the Collaborative International Pesticides Analytical Council Ltd, Hertfordshire, England. Copies available from Heffers Printers Ltd, King's Hedges Rd, Cambridge CB4 2PQ, England, 1985. 410 pp. Price: $77.00.

This handbook is an addendum to 3 previous ones published respectively in 1970, 1980, and 1983. It contains methods of analysis for 52 pesticide active ingredients, together with reagents and miscellaneous techniques. The methods described result from international collaborative studies and are approved by the Technical Committee at its annual meeting. A close cooperation with AOAC has resulted in adoption of many methods by both organizations.

Insect Management for Food Storage and Processing. Edited by F. J. Baur. Published by The American Association of Cereal Chemists, 3340 Pilot Knob Rd, St. Paul, MN 55121, 1985. 384 pp. Price: $55.00(members)/$65 (nonmembers). ISBN 0-913250-38-4.

This volume provides extensive information about avoiding, controlling, and eliminating insect problems. It is intended for sanitation professionals and pest control operators in all industries, not just the food industry, since many insect problems are encountered with consumer commodities such as paper and drugs. This volume emphasizes a systems approach for designing and using insect control methods. Regulatory matters, including those of EPA and FDA, are also discussed.

CRC Handbook of Mass Spectra of Environmental Contaminants. By R. A. Hites. Published by CRC Press, Inc., 2000 Corporate Blvd, NW, Boca Raton, FL 33431, 1985. 448 pp. Price: $69.50(U.S.)/$80.00(outside U.S.). ISBN 0-8493-0537-3.

This handbook is a collection of the electron impact mass spectra of 394 commonly encountered environmental pollutants. Each page is devoted to the examination of a single pollutant. All spectra are determined by analysis of data in EPA data bases. The major fragment ions are correlated with their respective structure. For all spectra, also given are the approved name of the Chemical Abstract Service, the common name of the compound, the article number given in the Merck Index, the CAS Registry Number, the molecular formula, and the nominal molecular weight of the compound.

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VIII QA in Sample Analysis
IX Records and Reporting
X Proficiency Testing — Inter- and Intralaboratory
XI Audit Procedures for QA
XII General Discussion and Review

COURSE SCHEDULE

Tuesday and Wednesday, July 8-9, 1986
Westpark Hotel, Arlington, Virginia

Tuesday and Wednesday, August 12-13, 1986
Westpark Hotel, Arlington, Virginia

Saturday and Sunday, September 13-14, 1986
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ASTM Standards on Precision and Bias for Various Applications. Published by The American Society for Testing and Materials, 1916 Race St, Philadelphia, PA 19103, 1985. 574 pp. Price: $19.20 (member)/$24.00 (nonmember). ISBN 0-8031-0457-X.

The precision and bias standards in this book will help to evaluate the precision of test measurements, develop in-house precision and bias for test methodology, develop research reports for interlaboratory test programs, improve in-house quality control systems, and keep up-to-date with the most current statistical terminology.

Control of Pesticide Applications and Residues in Food: A Guide and Directory. Edited by B. v Hofsten and G. Ekström. Published by Sweden Science Press, PO Box 118, S-751 04 Uppsala, Sweden, 1985. 300 pp. Price:SEK 240.00/U.S. $30.00. ISBN 91-86992-5.

The purpose of this book is to encourage international cooperation in the control of pesticides and similar compounds used for protecting food crops in the field and after harvest. Subjects covered include: introductory chapters written by international experts; information on international organizations and programs in the field of pesticide control; information on national authorities responsible for pesticide use, food safety, etc., in more than 80 countries; and references, maps, and an index.

Oxidation and Reduction in Organic and Analytical Chemistry. By A. Vincent. Published by John Wiley & Sons, Inc., One Wiley Dr, Somerset, NJ 08873, 1985. 83 pp. Price: $16.95. ISBN 0471-90698-0.

The aim of this book is to provide a self-instructional text to assist students in understanding the basic principles of equation balancing, mole calculations, and sample thermodynamics, which are fundamental to oxidation and reduction. It is a well balanced introduction to the topic, which begins with the basics and develops through to the more advanced areas. This book is therefore valuable both as an introductory and as a review text.

Handbook of Data on Organic Compounds. Edited by R. C. Weast. Published by CRC Press, Inc., 2000 Corporate Blvd, NW, Boca Raton, FL 33431, 1985. 1936 pp. (in 2 volumes). Price: $200.00(U.S.)/$230.00(outside U.S.). ISBN 0-8493-0400-8.

These volumes are a compilation of data on more than 24,000 organic compounds, presented in a number of useful formats. Volumes I and II contain an alphabetical listing of compounds, giving the following information, where applicable, for each: common names and synonyms, melting and boiling points, molecular formula and weight, line formula, refractive index, density, color, crystalline form, specific rotation, and solubility (greater than 10%). Since Beilstein and CAS numbers are given wherever possible, these references will serve as a means to more in-depth research.

INSTRUCTIONS TO AUTHORS

Scope of Articles

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

Preparation of Manuscript

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

Style and Format

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

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

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

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

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

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

Rev. 1/84

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The Journal The Journal of the Association of Official Analytical Chemists is published by AOAC, 1111 N 19th St, Suite 210, Arlington, VA 22209. The Journal is issued six times a year in January, March, May, July, September, and November. Each volume will contain approximately 1200 pages. The scope of the Journal broadly encompasses the development and validation of analytical procedures pertaining to both the physical and biological sciences related to agriculture, public health and safety, consumer protection, and the quality of the environment. Emphasis is focused on research and development to test and adopt precise, accurate, and sensitive methods for the analysis of foods, food additives and supplements, contaminants, cosmetics, drugs, toxins, hazardous substances, pesticides, feeds, fertilizers, and the environment.

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POSTMASTER: Send address changes to AOAC, 1111 N 19th St, Suite 210, Arlington, VA 22209.

REGIONAL AOAC MEETINGS

May 23, 1986 — **Eastern Ontario – Quebec Regional Section Meeting**
Sainte-Foy, Quebec
Contact: Gilles Paillard, Quebec Department of Agriculture,
2700 Rue Einstein, C2-72, Sainte-Foy, Quebec, Canada G1P 3W8, (418) 643-2561

June 16-18, 1986 — **Midwest Regional Section Meeting**
Lincoln, Nebraska
Contact: Tom Jensen, Nebraska Department of Agriculture,
3703 South 14th Street, Lincoln, NE 68502, (402) 471-2176

June 24-25, 1986 — **Northeast Regional Section Meeting**
Canisius College, Buffalo, NY
Contact: Gerald L. Roach, Food and Drug Administration,
599 Delaware Avenue, Buffalo, NY 14202, (716) 846-4494

AUTHORS IN THIS ISSUE

Banquet Address

Why I Am a Member of AOAC

JOSEPH P. HILE
Associate Commissioner for Regulatory Affairs, Food and Drug Administration, Rockville, MD 20857

You may not have noticed in looking through lists of previous Wiley Award winners that I, too, am a Wiley Award recipient. My Wiley Award is not from AOAC, however; rather, it is from the Association of Food and Drug Officials (AFDO). But like all Wiley Award recipients, I am very proud of this honor, and I see it as one of the highlights of my career. This is particularly true of both Wiley Awards because they are presented by one's peers. Unfortunately for me, the AFDO award does not carry a stipend of $2500 along with it. I would think that AOAC, in view of its policy of harmonization of procedures between sister associations, would harmonize the Wiley Awards with AFDO so that both awards carried the $2500 stipend, and that AFDO would make it retroactive!

You also might be surprised to learn that in the Wiley tradition, I am a member of AOAC—a member not solely by virtue of my position at the Food and Drug Administration (although that makes me eligible for membership), nor solely by virtue of paying $25 a year in dues, although that makes it official. It is because I believe in the objectives of AOAC, I recognize its importance, and I am committed to keeping it strong. It is important that I can speak to you not as a guest but as a fellow member of *our* association as it begins its second century of organizational life. It is also my chance as a member to give you some specific insights about why I continue to pay my dues, what I believe I can give to the organization, and what I expect the organization can give back to me.

I am a member of AOAC because I believe in its basic goal: an association of scientists and science administrators dedicated to bringing order to the world of analytical methods—methods that have been studied in a variety of laboratory settings; methods that are suitable and have the accuracy and precision to be used as standards in a legal setting; methods that have been validated and peer-reviewed; methods so certain in their validity that I am willing, as the Associate Commissioner for Regulatory Affairs, to recommend in a court of law that a person's freedoms be restricted or that property be seized on the basis of the result.

The time-tried procedure employed by our association to prove the worth of analytical methods has been invaluable. In the first place, it has prevented the use of inaccurate methods and thus has prevented unfairness. It has given industry confidence that the methods it uses are dependable, and in enforcement operations it has provided a seal of approval that courts and juries respect. Certainly, it permits the analyst to fulfill his or her obligation to use only methods that have been established as accurate, precise, and capable of giving reproducible results in the hands of competent scientists. The current edition of *Official Methods of Analysis* attests to the progress that the Association has made in keeping the methods up to date and meeting the ever-increasing demand for new methods. This achievement over the years has been made possible only because of the devotion of our colleagues to this lofty, self-imposed task. It is from this firm foundation, then, that I look to the future and pose the question: What can AOAC and I do for each other?

Most of us learned long ago that practical enforcement of food and drug laws rests in large measure on the factual evidence supplied by the results of scientific analysis. But advances in technology and changing societal expectations bring about amendments to these laws that, in turn, bring new regulatory challenges. It is imperative that our methods of analysis keep pace with these changes. The problem is how to accomplish that. Should we expect that all methods published by AOAC meet the extremely high standards for a recommended method? Our late friend and colleague of the United Kingdom, Dr. Harold Egan, answered the basic question for us some years back when he forcibly reminded us that only those methods that have been fully evaluated and collaboratively studied can become *recommended* methods. Therefore, I hope that we will always reserve publication in *Official Methods of Analysis* for only those definitive methods that, after exhaustive investigation, are recommended methods found to have no known bias or ambiguity and that provide a result that is the best known approximation of the true value. The problem is that we cannot always wait for the process before testing the method in real life. I am pleased, therefore, that AOAC, through its journal and other publications, provides what I would describe as generally accepted operational methods. These methods reflect the fact that the Association is forward-looking and that we are attempting to keep abreast of the technological changes of our world.

Meantime, while they are not yet recommended methods, they have great utility for monitoring and screening.

Regardless of the stage of acceptance of a method, however, as science and technology advance, there is a tendency for the methods to advance in their sophistication, sensitivity, and ability to detect. We in AOAC should be cognizant that chasing "zero" detection is not always desirable, necessary, or appropriate. The methods developed by analysts and accepted by AOAC must be practical as well as technologically sound and simple enough that both large and small laboratories, in either the private or the public sector, can use them with ease and confidence. Not all laboratories are able to purchase the most sophisticated and expensive equipment, and the methods recommended by AOAC should not, if at all possible, pose an undue burden on the budgets of smaller firms or governments. Ideally, the methods recommended by AOAC should assure that necessary materials, reagents, and equipment are readily available from more than one source.

As I mentioned, I expect the Association to protect the *Official Methods of Analysis* and to continue to provide the approval process that assures expert peer review of every method that is adopted as official first or final action. Obviously this peer review must in truth be fair and not colored by the selfish interests of any one laboratory, whether it be government, industry, private, or academic. The method must result in due process so that industry and local governments, for example, do not feel that the federal government is imposing methods on the non-federal chemist. Equally important, the process must be open to public scrutiny so that our critics can have confidence that there is no chicanery among the Association's membership. I would also like to suggest that AOAC study the process used by the United States Pharmacopeial Convention for soliciting public comment on its methods. It may not work for AOAC, but it should be worth a try.

On the other hand, as a member, I expect to do my share in advancing the work of the Association. Since I am a science administrator with some 22 widely diversified laboratories under my supervision, I find that one of my most important contributions is in motivating my organization's scientists to become actively involved in AOAC—to serve in various capacities as Associate and General Referees, officers of sections, and chairs of committees.

Let me digress a moment. The regional section concept is an ideal way of attracting interested analysts into our organization. It provides an opportunity for them to participate in AOAC activities and interact with their associates, even if they cannot always—or perhaps ever—attend the international meetings. I believe the establishment of regional sections should be a top-priority initiative for all of us.

To return to the subject of my obligations, I should expect to provide my share of the analytical resources necessary to carry out collaborative studies. In this regard, however, my success rate raises two matters of concern. First, if one simply counts the number of collaborative studies that result in official methods, one finds that the overwhelming majority of participants come from Food and Drug Administration and industry laboratories. Therefore, I must challenge all of you to invite, in any way you can, wider participation in our program by the university laboratories and engender a reaffirmation of the value of full participation at the state and local government levels.

Second, I am encouraged by the greater role that the regulated industry is playing in the Association, for it is absolutely critical to AOAC that its credibility be enhanced by the widest possible participation in the process. Therefore, because the industry is acting as a full partner, I urge the Association to consider broadening industry's role in AOAC by doing away with associate membership and extending full membership privileges to all industry scientists. The increase in international trade, as well as improved modes of communication, suggests that the current initiatives to involve the international scientific community as an integral part of AOAC should be strengthened in future years. Science, more than any other communication medium, overcomes any international language barriers.

Finally, both AOAC as an organization and I as a member must be forward-looking in our expectations of one another. AOAC methods must be up to date; to the extent that our budgets allow, our laboratories must be able to take advantage of technological change; our AOAC methods approval process must respond in kind.

For example, the field of biotechnology represents such a challenge, and we must begin to deal with it today. One of the products of the new biotechnology that will surely facilitate our work is the gene probe. These probes permit us to detect pathogenic microorganisms in foods because they bind specifically to the microorganism DNA. The genus and species of a microorganism is assuming less and less importance in judging pathogenic potential, while the ability to produce a toxin or to be invasive is becoming the real issue. The development of genetic probes to detect these abilities has already begun.

To date, FDA's experience with such gene probes has been successful. The basic methodology has been tested collaboratively and accepted by AOAC, and FDA has trained its field personnel in these techniques. Our pathogen surveillance programs for several foods are already applying gene probes to differentiate nonhazardous *Escherichia coli* in foods from those capable of causing human disease. Now we are expanding our capabilities in this area to include *Yersinia, Campylobacter, Listeria,* and other pathogens of concern. It is noteworthy that FDA's Center for Devices and Radiological Health has recently approved for clinical use the first DNA probe used for detecting *Legionella*. FDA hopes that this technology will also enable us to be more effective in those areas in which our current methods do not work well, namely, in finding human viruses in foods.

AOAC's ability to help develop and establish these new methods will strengthen all of our efforts to assure safe and wholesome products in the future.

At the beginning of my remarks, I listed several reasons why I am a member of AOAC. Clearly, another reason is tradition. It would be hard to work for the Food and Drug Administration and not be caught up in the mystique of Harvey Wiley—a mystique that is reflected every day in the dedication to the job that is so typical of all of us who work in the food and drug area. With tradition goes pride: pride in the accomplishments of one's friends and associates who have carried on the Wiley tradition. My regards go to the myriad of persons in FDA, state laboratories, Canadian agencies, and others, too numerous to list here. These persons have been or are my partners in assuring consumer protection through fair and equitable law enforcement. All AOAC members are part of that partnership. You share with me the responsibility of assuring that AOAC continues its rightful role and enters strong and viable into its second century of service. In the Wiley tradition, I know we can do it.

HARMONIZATION OF COLLABORATIVE ANALYTICAL STUDIES: SECOND INTERNATIONAL SYMPOSIUM

October 25–27, 1984, at the
National Academy of Sciences, Washington, DC, USA

This second International Harmonization Symposium is dedicated to Harold Egan, the initiator of this program, who passed away June 28, 1984. The symposium was sponsored jointly by the Analytical Chemistry, the Applied Chemistry, and the Clinical Chemistry Divisions of the International Union of Pure and Applied Chemistry (IUPAC), the U.S. National Committee for IUPAC, and the Association of Official Analytical Chemists (AOAC), on the occasion of the Centennial of AOAC (1884–1984).

Characteristically, Dr Egan had already prepared his introduction, which is given as the first paper of the Symposium. Dr Egan's plans were continued and executed by L. Coles, H. Frehse, and W. Horwitz.

A conclusion reached by the attendees was to have IUPAC continue the efforts toward harmonization. For this purpose, the next formal meeting, at which time an attempt will be made to actually harmonize protocols, will be held in Geneva in the spring, 1987.

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Method Validation: The Harmonization of Collaborative Analytical Studies

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The first International Symposium on the Harmonization of Collaborative Analytical Studies was held in Helsinki, August 20–21, 1981 (1, 2). This was in fact the first occasion on which many of the international organizations which publish compendia of analytical methods developed by the process of collaborative analytical studies had met to compare philosophies. The meeting attendees urged the presidents of the 3 sponsoring IUPAC divisions to give positive attention to the need to develop international guidelines, to harmonize definitions of the basic parameters concerned and the philosophy of applying these to the validation process, and to provide a continuing forum for the exchange of information in these fields. If nothing else, the symposium aroused a critical awareness that many different international groups were working, largely independently, in a field of fundamental and sometimes far-reaching significance in food, health, environmental quality, commerce, industrial specification, and control. At the same time, it is appreciated that analysis is often only an index of biological or other function and, for this reason alone, it is important that, in areas where approximations and uncertainties abound, the reliability of the analytical aspects be properly understood and matched to their purpose. Finally, there is an economic factor in terms of time, money, and professional resources, which calls for a need to maximize the application of these in a world ever more demanding of analytical services.

The first symposium also stimulated further reflection on the present position and the different functions on which interlaboratory collaborative study exercises are based. A main distinction can be drawn between the uses of a study to determine the attributes of a method for acceptance purposes and for performance purposes. A study may also be used to compare the attributes of several methods, to compare the ability of different analysts, or to establish a consensus value for a reference material. Consideration of these aspects pointed to the need, more than ever, for criteria for the acceptance or validation of methods, particularly those used by enforcement authorities with the implicit need to maintain a sometimes delicate balance between fraud or hazard and usefulness and value. The challenge has been taken up in particular by the Association of Official Analytical Chemists, which decided, as part of its centennial arrangements in 1984, to establish a definitive protocol for the validation of methods by the process of collaborative interlaboratory study. The final first draft of this document was published last year (3). There is no illusion that this is a perfect framework for validation, and it is clear (and perhaps always has been clear) that total harmonization is neither practicable nor indeed necessarily desirable. Two parts to the AOAC strategy, which have developed at different paces, can clearly be distinguished: the basic experimental framework and the manner in which results obtained using the framework are interpreted. Both of these aspects will be discussed further at this second Harmonization Symposium, which is sponsored jointly by IUPAC, AOAC, and the U.S. National Committee for IUPAC.

The objective of the second symposium will be to identify the design essential for validation of the performance of analytical methods, to identify where possible the minimum criteria for an interlaboratory study for validation in the light of this, and to compare the various factors and criteria identified in the design, as recognized by the various international and other organizations experienced in this field, many of which will be represented at the symposium, with the view where possible of harmonization on an international basis.

John Taylor has identified a hierarchy of analytical methodology (4) in which the term "protocol" is seen as a set of definitive, mandatory directions, to be followed without exception. At the same time, he has recognized that it is impossible to describe each and every step with equal, not to say complete, absence of ambiguity or scope for individual interpretation. The aim of a method description must be to minimize the variability which can arise from this cause and it is partly for this reason that the concept of ruggedness has been introduced into the AOAC protocol referred to above. This raises wider issues such as the validation of the analyst who performs the method, or the accreditation of the laboratory systems in which the analyst is working. All of these enter into the real world of applied analysis and for this reason the organizers of the second symposium introduced a workshop element into the program. In this way, it is hoped to gain a fuller insight into some of the practical problems that have been encountered, with a "how to do it" element included.

Effective harmonization means that one organization can judge whether the validation of a method by another organization can be accepted without further testing; this would be to the advantage of all concerned.

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[1]Deceased.

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Harmonization of Collaborative Study Protocols

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The 2 major protocols for the design, conduct, and interpretation of collaborative analytical studies—those from AOAC and the International Organization for Standardization—are already fairly well harmonized. The statistical models are identical and the outlier tests are essentially the same. The major differences are in symbols and terminology and in the specification of the minimum number of laboratories and replicates.

To harmonize the conduct of collaborative studies, it is necessary to know where disharmony exists. A chart (Table 1) was prepared to discover the points of difference between the 2 major protocols for the design, conduct, and interpretation of collaborative studies. The AOAC column was obtained by interpreting the *Statistical Manual of the AOAC* (1), which was supplemented by the latest reports of the AOAC Committee on Collaborative Interlaboratory Studies after revision to include the Dixon test as used by the International Organization for Standardization (ISO) (2, 3). The ISO protocol is *ISO 5725-1981* (4).

When arranged in this manner, the 2 protocols are remarkably similar. The major differences are in matters of symbols and terminology, which are readily remedied.

Both organizations have the same understanding of the term "collaborative study," with one minor difference. The ISO protocol is restricted by its title to obtaining precision indices only. Consequently it is directed primarily toward those specifications that are defined by the method itself. This restriction created the amusing situation in which the organizers of an ISO collaborative study prepared materials with known compositions but refused to report recoveries of added analyte because ISO 5725 made no provision for this important parameter. However, in such cases it is often an easy matter to calculate recoveries from the reported data, since reports of studies using the ISO protocol usually indicate how materials for the collaborative study were prepared.

There is a minor difference with respect to the point at which the collaborative study is performed in the development of a method ("standard" in ISO terminology). AOAC makes the successful completion of a collaborative study the prerequisite for the adoption of a method. ISO performs the precision experiment "once the standard has been established." This difference in application has no bearing on the statistical analysis of the data. The net result is that ISO committees, in food analysis at least, have been surprised to discover that their approved standards have considerably poorer precision than was assumed at the time of adoption.

Experimental Design

The same experimental design is used by both protocols: q materials (levels of analyte) are sent to p laboratories, which perform n tests (replicates) at each level. The symbols used here are those of ISO 5725. As an aid to memory it would be better to use m materials sent to l laboratories to perform r replicate tests, but the symbol r is used later for repeatability. Both protocols recognize the split level design (designated as the "Youden matched pair" by AOAC) in which each level is split into 2 levels, which differ only slightly from each other and are analyzed only once at each level.

Both protocols are harmonized with respect to the following auxiliary points: Laboratories are selected at random from the population of qualified laboratories; identical portions of homogeneous materials are submitted to all laboratories; no instructions beyond the written method are supplied to the analysts during the study unless special instructions are supplied to all analysts; the use of training materials is encouraged prior to the main study; and analysts are instructed to report all test results, including those that are discordant, regardless of whether they are used in the statistical analysis.

There are some differences with respect to the minimum numbers recommended by the 2 organizations. For the number of materials (levels), AOAC recommends 5 or 6; ISO, 6. For the number of laboratories, AOAC recommends 6 or 5 so that the number of laboratories times the number of materials equals at least 30; ISO recommends at least 8 laboratories with no comparable combination (laboratories × materials). AOAC will accept single analyses (no replication), obtaining the within-laboratory precision through the use of the split level design; ISO requires a minimum of 2 replicates to obtain repeatability directly but also permits the split level design. The practical effect of these differences is small because most AOAC studies exceed the minimum requirements. AOAC could accept an ISO study which meets ISO minimum requirements, but ISO might not accept an AOAC study which uses AOAC minimum requirements.

With respect to repeatability (*within-laboratory* variability), ISO requires the performance of 2 replicates for every test. ISO apparently does not recognize that the use of parallel duplicates usually leads to underestimation of within-laboratory variability. Youden perceived this problem and recommended elimination of parallel replicates in favor of the split level design. Repeatability can always be obtained through replication by an individual laboratory, independent of a collaborative study. However, the between-laboratories variability is the most important precision-related parameter obtained from the collaborative study, and it cannot be obtained in any other way. Therefore, if the resources that can be assigned to a collaborative study are limited, it is more important to obtain between-laboratories data than within-laboratory data. There is no objection to the performance of parallel duplicates if they do not inhibit the gathering of the more important between-laboratories information. The use of blind duplicates or the split level design in place of parallel duplicates should be encouraged, since these designs permit the most efficient use of resources to obtain the important statistical parameters.

Statistical Model

Both organizations use the same statistical model, which is crucial from a statistical point of view. The model is as follows:

$$y = m + B + e \qquad (1)$$

where y = a single test result, m = the "true" mean, B = the laboratory deviation from m, and e = the random error in each test result. Because the laboratory term B is made up of 2 components, a systematic component, B_s (the bias of the

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method), and a random component, B_e (the bias of the laboratory relative to the bias of the method), substituting these components into equation (*1*) shows the contribution of systematic and random effects to a given test result:

$$y = m + \overbrace{B_s + B_e}^{\text{Systematic}} + \overbrace{e}^{\text{Random}} \qquad (2)$$

The variance components are estimated the same way. The variance of the laboratory averages, var(B), is as follows:

$$\text{var}(B) = \text{var}(B_s + B_e) = \text{var}(B_s) + \text{var}(B_e) \qquad (3)$$

By definition the variance of a systematic component is zero since it is a constant additive term, so

$$\text{var}(B) = 0 + \text{var}(B_e) = \sigma_L^2 \qquad (4)$$

where σ_L^2 is the variance of the laboratory averages, which is *estimated* from the collaborative study as s_L^2. Although both protocols use the standard statistical symbols of "σ" to refer to the true or population standard deviation and "s" to refer to the estimate of this value, harmonization is needed in the use of subscripts.

The estimate of variance within any laboratory, i, is given by $\text{var}(e_i) = s_i^2$. The average of all the within-laboratory variances from all the laboratories in the collaborative study is the common repeatability (r) variance, $\text{var}(e_i)$, which is assumed to be applicable to all laboratories:

$$\text{var}(e_i) = \sigma_r^2 \qquad (5)$$

The term σ_r^2 is estimated in the collaborative study as s_r^2, which is the repeatability variance that is used by both ISO and AOAC, although AOAC uses the symbol s_o^2.

The most important precision parameter is the reproducibility variance, s_R^2, which is estimated by combining the within-laboratory and the between-laboratories variances:

$$s_R^2 = s_r^2 + s_L^2 \qquad (6)$$

AOAC uses the symbol s_x^2 for the same composite quantity. Both protocols use the symbol s_L for the same term with the same meaning ("pure" between-laboratories standard deviation, i.e., without the within-laboratory component).

Statistical Analysis

Both organizations begin the statistical analysis by identifying and removing outliers, a matter which is discussed

Table 1. Comparison of features of AOAC and ISO protocols[a]

Feature	AOAC	ISO
Status of method tested:	Optimized initial test	Standardized final test
Design		
q materials or *q* levels of analyte sent to *p* laboratories, which perform *n* tests at each level; or each level is split into 2 sublevels, which differ only slightly from each other and are analyzed once at each level	X	X
Identical material analyzed by all laboratories	X	X
Short interval of time for repeatability	X	X
Minimum number of		
levels	5 or 6	6
presented blind	X	—
presented as split level	X	X
laboratories	6 or 5	8
selection	random	random
levels × laboratories	30	—
replicates	1	2
Further instructions to analyst during test	no	no
Use of training materials	X	X
Report all tests results	X	X
Statistical model: $y = m + B + e$	implied	X
m = mean; B = laboratory deviation from m; e = random error in each value		
Bias (= difference from true value)	X	—
$\text{var}(B) = \sigma_L^2$; $B = B_s + B_e$, where B_s is a systematic component and B_e is a random component	X	X
$\text{var}(e) = \sigma_o^2$ (σ_r^2) = within-laboratory variance; the common repeatability variance applicable to all laboratories	X	X
$s_R^2 = s_L^2 + s_r^2$	X	X
Statistical Analysis		
Identify and remove outliers	X	X
Calculate s_r, s_L, and s_R for each level	X	X
Calculate RSD_r and RSD_R for each level	X	—
Calculate repeatability and reproducibility for each level: $r = 2.83\ s_r$; $R = 2.83\ s_R$ (2.83 is the factor for duplicates)	—	X
Determine relationship between m and r, R, RSD_r, and RSD_R	—	X
Use set of transformed values of R that gives best linear fit with m	—	X
(1) Proportional $R = vm$		
(2) Linear $R = u + vm$		
(3) Logarithmic $\log R = c + d \log m$		
where u, v, c, and d are constants		
Treat missing data	—	X
Outliers		
Critical levels of 1 and 5%	X	X
Discard at 1%	X	X
Retain at 1–5%	—	X
Cochran test (for extreme variance of individual laboratories) (1-tail)	X	X
Dixon test (for extreme values of laboratory averages) (2-tail; alpha = 0.01 overall)	X	X
Grubbs test (for extreme values of laboratory averages) (2-tail; alpha = 0.01 overall)	exptl	—
Rank sum test (consistent systematic laboratory deviation)	X	—
Influential outlier test (removal results in decrease in RSD_R of 40% or more)	exptl	—
If estimate of s_L^2 is negative, set $s_L^2 = 0$	X	X

[a] X = Yes or statement present; — = No or no statement present. A number of statistical points have been simplified for this chart.

separately. They both calculate the same fundamental precision parameters in the same way from the estimates of the variance, s_r^2 (s_o^2), s_L^2, and s_R^2 (s_x^2). They both calculate the relative standard deviations (coefficients of variation) in percentages by dividing the standard deviations by the mean concentration and multiplying by 100, although AOAC places considerably more importance on this parameter than does ISO. The maximum tolerable difference parameters (ISO's r and R), quantities unfamiliar to AOAC practitioners, are emphasized by ISO.

The ISO terms repeatability (r) and reproducibility (R) are very useful and practical quality control parameters expressing the maximum difference that would be expected with 95% probability between 2 values from the same laboratory reported by the same analyst (repeatability) and between 2 values from different laboratories (reproducibility). This method of expression was advocated by E. F. Schultz, Chairman, AOAC Committee on Statistics, in a presentation to AOAC Associate Referees at the 1968 annual meeting, but it was never acted upon.

AOAC favors the use of the relative standard deviation (RSD) because in much of its work this parameter is relatively constant over several orders of magnitude. Over a short range of concentrations, the ISO parameters are also constant. These 2 viewpoints are not at all incompatible. They merely reflect a history of involvement in different commodities and situations. In this connection, the ISO document attempts to fit an equation that relates the precision parameters to the mean concentration for use over the entire range covered by the interlaboratory study. Some suggested ways of relating the precision parameters to the mean are through the use of transformations: proportional, linear, or logarithmic.

In addition, the ISO document specifically indicates that it does not discuss the confidence bounds of the precision parameters. Our research in this field of variability indicates that the variability of variability is so large that it is probably unprofitable to seek a relationship between the precision parameters and the mean concentration from the values developed in a single collaborative study.

Outliers

No topic in collaborative studies generates more controversy than the matter of handling outliers. The positions taken with respect to outlier removal vary from one extreme of not allowing outlier removal unless an explanation is found for the aberrant value to the other extreme of removing values until a normal distribution of the remaining values is attained. A substantial fraction of collaborative studies contain outliers, which are defined by Healy (5) as observations that depart from expectation to an improbable extent. However, in the context of a collaborative study, we have found that many outliers are innocuous. Such noninfluential outliers may meet the conventional criteria of statistical significance for removal by the Dixon and Cochran tests, but after their removal neither the mean nor the standard deviation (or related parameters) is changed appreciably. In this context an appreciable change is a reduction of approximately 40% in the relative standard deviation. However, these changes can be quantitated more precisely by relating them to the Grubbs test for outliers.

At the present time, both ISO and AOAC apply the same outlier tests—the Dixon test for extreme values and the Cochran test for extreme variance. AOAC recently changed its Dixon test to that used by ISO, so the outlier tests are now the same. AOAC leaves considerable latitude for removal of flagged outliers to the administrator of the study; ISO removes only those flagged as outliers at the 99% significance level.

Although outliers appear in a substantial fraction of all collaborative assays and their appearance generates most of the discussion about the interpretation of the study, they should be put in proper perspective. In perhaps as many as 90–95% of all collaborative assays no outliers are present or the outliers need not be removed because they are unimportant; in perhaps 2–4% of the assays almost everyone agrees that the outliers which appear are so gross that they must be removed. This leaves at most a few percent of the assays or data about which reasonable, experienced interpreters of collaborative studies may disagree as to their treatment. In such cases, the study should probably be repeated, if the disagreement extends to the conclusion of acceptance or disapproval of the method.

Conclusion

At the present time we have protocols which are essentially harmonized or which will give the same interpretation in practically all cases, whether the study is conducted by ISO or AOAC rules. The discrepancies are in the "straggler" region. Once this area of inconsistency is resolved, we will have achieved harmonization for all practical purposes.

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Parameters and Definitions in Harmonization of Collaborative Analytical Studies

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IUPAC, AOAC, and ISO participate in developing, validating, and adopting analytical methods for international use. Harmonization of the different interests of these organizations is desirable, as is harmonization of parameters of precision of test methods. ISO Technical Committee 69 is responsible for standards that deal with statistical interpretation of test results and data, sampling procedures, and precision of test methods.

This second International Harmonization Symposium is dedicated to the late Harold Egan, our valued colleague, who did so much for the success of this symposium and who proposed the following as a practical starting point for the discussion:

> "The number of materials (samples) examined times the number of participating laboratories should be at least 30, with a minimum of 5 laboratories providing usable data from single determinations."

Dr Egan's proposal relates to the validation of a candidate method through a standardization experiment. Let us accept his advice, which we may now also regard as a legacy.

John Taylor's "Validation of Analytical Methods" (1) may be another starting point for our discussions. His "hierarchy of analytical methodology" can be used, for example, to show the consecutive grouped interests of different organizations. A general demarcation can be made, and concepts and aims can be separated accordingly:

Hierarchy	*Main interest*	*Validation*
Technique	IUPAC	
Method	IUPAC AOAC (NSB)[a]	standardization experiment
Procedure	AOAC (NSB) ISO (O/RB)[b]	precision experiment
Protocol		

[a]NSB denotes national standardization bodies.
[b]O/RB denotes official/regulatory bodies.

IUPAC's role and interest begins with *Technique,* at which level it has a leading role in developing scientific principles and in basic research.

The next level is *Method.* IUPAC remains interested in many methods, in the specific adaptation of a technique. This is probably the level where AOAC interest and that of national standardization bodies (NSB) begins—the selection of candidate methods.

The *Procedure* level, which follows, is the level of written directions necessary to a method. This is the level where standardization experiments are made to validate a selected method. Here IUPAC interest is much lower; however, it is still manifest in a few directions (for example, analysis of oils, fats, and derivatives; atmospheric pollutants; pesticide residues; etc.). At this level AOAC and the national standardization bodies are very active and ISO (International Organization for Standardization) interest begins. There are in fact ISO technical committees (for instance ISO/TC 102 "Iron ore") which begin their activities even earlier—at the *Method* level.

Finally, we reach the *Protocol* level, the set of definitive directions that must be followed without deviation if the analytical results are to be accepted for a given purpose. This is the domain of official/regulatory bodies (mainly national, sometimes regional or international) and it is certainly the domain of ISO.

In short, IUPAC, AOAC, and the national standardization bodies set rules for almost the entire process of developing analytical methods, while ISO is mainly interested in the adoption of methods already standardized as international (reference) methods and in organizing the *precision experiment* for them. If this hierarchy and these main interests are to some extent well grouped, there is no need to seek interchangeability of procedures aimed at different purposes. Some harmonization for the transition between levels is, however, desirable.

It is evident that the best solution would be for procedure-type documents to be adopted as protocol-type documents as well. There would then be no need to repeat validation procedures. Common sense dictates that we should all make efforts to achieve this goal.

In ISO, the preparation of international standards is decentralized and is performed in the various specialized technical committees. About 30 of them set up standards for chemical analysis in their respective fields.

The main technical committee that prepares exclusively analytical standards is ISO/TC 47 "Chemistry." This technical committee has published a basic document, ISO 78/2-1982 (2). This standard has been accepted or is followed by other organizations as well. Its clause 14.2, Precision, states that ". . . It is essential to indicate the precision data. . . ." In fact, a considerable proportion of ISO analytical standards does not yet give these data. The reason for this is the way a method is accepted within ISO as an international standard.

In the regular way, the proposer of a new work item offers one or more existing standardized methods (national standard or a standard from a specialized agency). These, very often, have precision data obtained in interlaboratory studies performed usually by leading laboratories. In the course of the preparation of international standards, members of the relevant technical committees, as well as international organizations having liaison status with ISO, may propose practical modifications or express a wish to redefine the details of the method in a protocol-like style. The proposals are circulated, commented on, and discussed in meetings where specialists can evaluate all the details. Finally, through a multi-stage voting procedure, the method is accepted by ISO Council as an international standard.

There is usually a second phase in which precision experiments are organized to obtain the performance characteristics of the already published standard method. The precision data can be included at the next revision of the standard.

This is the historical approach and it is also a logical one, provided that the procedure adopted has well documented, traceable performance characteristics and that the modifications introduced during the adoption have not gone beyond the ruggedness of the original procedure. In a few cases when that has happened, precision experiments have given disap-

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pointing results. Modifications are often useful, especially when they make the description more detailed and more easily understandable, "protocol"-like, as John Taylor terms it.

International standards are expected to be used globally, including use by personnel in developing countries where climatic and other circumstances have an influence on the precision of the method. Therefore, when data from a global experiment are analyzed, special care should be devoted to stragglers and outliers, in evaluating all influencing factors.

When selecting laboratories for a study, ISO technical committees try not to exclude those working with average equipment and resources. The first class laboratory featuring all the latest equipment is not typical. We should not in any case forget that it is not necessarily the superior method that shows the best results in a trial but the one that works consistently with the same error throughout a wide range of laboratories!

All this gives the background to *parameters and definitions* which we should look at in the practical and whole context.

Parameters

Important parameters of precision of test methods in ISO 5725-1981 (3) are repeatability, reproducibility, and outliers. ISO 5725 is now being revised in 2 steps. A minor revision has been prepared (4) as the first step. To improve clarity and to harmonize with AOAC, a few changes have already been introduced. Standard deviations of repeatability (s_r) and reproducibility (s_R) as precision measures as alternatives for r and R have been accepted and incorporated.

As a further step to harmonization, William Horwitz has proposed the addition of another precision parameter, the coefficient of variation (CV), known also as relative standard deviation (RSD). In fact, in the chemical field this is a very important parameter because CVs are constant over a large range of concentrations.

Another proposal of Dr Horwitz that also deserves full attention concerns terminology. He mentions that some of the difficulties that are being experienced in applying and understanding ISO 5725 would be dispelled if the values calculated and used by this standard ($2 \times \sqrt{2} \times$ the appropriate standard deviation) were termed "repeatability interval" and "reproducibility interval." This would permit the use of the terms repeatability and reproducibility with standard deviations, relative standard deviations, and coefficients of variations, whose use may be preferred by other organizations. This would result in better communication with other interested organizations for the benefit of all.

The problem of *outliers* is so complex that strict rules cannot be laid down for individual technical committees and even less for ISO as a whole. Nevertheless, because of the general nature of ISO 5725, *guidance* concerning outliers should be given. The arbitrary 5% and 1% significance levels are adopted in ISO 5725 for identifying *suspicious analytical data,* not for eliminating laboratories. A certain amount of subjective judgment should be included in any workable system.

Dr Horwitz mentions the concept of "influential outliers." According to this, the removal of these would result in about 40% improvement in the precision parameters. His proposition, together with the generalized Grubbs outlier test, will certainly be taken into consideration at the time of the second revision of ISO 5725. Richard Albert's discussion at this symposium is devoted entirely to dealing with outliers, and we hope to conceive ideas which may also be helpful for improving our policy with regard to them.

ISO 5725, in fact, does not hunt for outliers. In collaborative analytical studies these do sometimes occur and, if not eliminated, they can cause a marked increase in the estimates of repeatability and reproducibility. When evaluating an international collaborative analytical study the panel of experts will decide what to do with laboratories whose results can be regarded as outliers.

But here as well, there should be no contradiction between AOAC and ISO insofar as AOAC is concerned with standardization experiments and with getting the best precision the methods can furnish, while ISO is concerned with precision experiments and should demonstrate the possibilities of a method when applied globally, average laboratories included.

Definitions

The ISO Technical Committee TC 69 "Application of Statistical Methods" is responsible for a number of international standards that deal with statistical interpretation of test results and data, sampling procedures, and precision of test methods. These standards include definitions where appropriate, and it should be noted that ISO 3534 (5) is specially devoted to terms, definitions, and symbols used in the statistical field and that the terms and definitions are given in parallel, English and French. TC 69, which can act as an advisor to other ISO technical committees, has prepared its definitions with a view to their being used to harmonize specific needs elsewhere. There are about 30 ISO technical committees that can benefit from the TC 69 work as well as other international organizations, but the adoption of what may be slightly new approaches is not always easy.

The main drawbacks are the tolerance of synonyms in technical literature and the multiple meanings of some terms. Unfortunately the random choice of terms is deeply rooted in science and can quite often cause misunderstanding. The standardization process, however, has the virtue of identifying these terminology problems and standardization of terms in more than one language, as in ISO deliberations, often indicates unexpected sources of trouble. A few examples are as follows:

> The English "repeatability" has 2 French equivalents: "répétabilité" and "fidélité," while French "fidélité" has 2 English equivalents: "repeatability" and "precision."
>
> The English "bias" is, in French, "biais" or "justesse," while the French "justesse" is in English "bias" or "accuracy of the mean."

In these cases ISO 3534 establishes parallel terms throughout, the rare synonyms always occurring with the same definition.

There are cases where the terms can be even more misleading than useful, for example the term "accuracy." Unfortunately this word is often used interchangeably (and incorrectly) with "precision." This should be avoided, and possibly we should deprecate the use of "accuracy" even in composite expressions.

We hope that the present symposium will help the interested parties to achieve a better harmonization of collaborative analytical studies. In ISO the relevant committee will take into account the results of this symposium.

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Role of Collaborative and Cooperative Studies in Evaluation of Analytical Methods

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A method proposed as a standard or for use in a regulatory process must be reliable and its typical performance characteristics should be stated and verified. Collaborative testing is the most acceptable way to accomplish the latter but its function should not be misunderstood. Such testing can verify performance characteristics and experimentally demonstrate that the methodology can be used successfully by a representative group of laboratories. It does not necessarily support the validity of any data obtained using the method because this may depend on many other factors including the expertise of the laboratory and the quality assurance aspects of its measurement process.

Cooperative or collaborative studies are those in which a number of analysts participate to test their analytical proficiency, to establish consensus values for parameters of products or materials, or to evaluate measurement methodology. The discussion which follows is directed largely to the latter of these kinds of studies. However, there are similarities in all of these activities and it is my intention to extend the ideas presented here as appropriate to the other 2 kinds in another publication.

Methods and procedures for conducting collaborative testing are discussed extensively in a number of publications (1–6). Accordingly, this paper will not consider the mechanics of such testing but will be directed to the significance of collaborative test data when applied to the evaluation of methodology.

Why Collaboratively Test?

Collaborative testing is generally considered to be a basic requirement for method standardization. A standard method is essentially useless unless its operational characteristics have been evaluated and defined precisely and it has been demonstrated to be useful for its stated purpose. When methods are specified for regulatory purposes, there is an even greater requirement that their reliability be confirmed and well documented. Because use of limited information, and even a single laboratory's opinion on such matters, can be unreliable, the pooled experience of representative potential users is considered necessary. Thus the need for collaborative testing has arisen.

A little reflection and even the recall of bitter experience in some cases will convince most readers that specific performance characteristics are not inherent for a method of measurement. The analyst, the laboratory environment, the equipment used, and the quality control exercised are very influential on the precision and accuracy attained at any time of use (7) so that one cannot say categorically that a given method has a specific precision or limit of detection, for example. One can say only what was experimentally found under certain conditions, but there is no way to predict whether a given user will duplicate that experience or obtain better or worse results. Even with such limitations, collaborative testing is considered to be an invaluable aspect of method validation, as will be discussed here.

Information Provided by a Collaborative Test

The most important decisions that must be made when selecting or evaluating methodology are as follows:

(*1*) Is the method technically sound?
(*2*) What are its scope and limitations?
(*3*) Are typical statistically supported performance characteristics presented to provide a basis for considering its applicability for a given use?
  a. Precision
  b. Biases
  c. Detection levels
  d. Sensitivity
  e. Useful range
(*4*) Is the description—style, format, rhetoric—sufficiently clear so that it may be used by the audience to which it is addressed?

The first 2 decisions are outside the scope of collaborative testing, yet they must be made before any use or testing of a method or procedure. The world of science is the arena that provides the information base on which such decisions can be made.

The values listed in (*3*) may be called "figures of merit" useful for typifying methodology. Figures of merit are essential for *selection* of methodology but they do not measure *performance* in a specific application. Only the performance statistics obtained in the course of a specific application can be used for this purpose. Figures of merit are obtained best by a peer laboratory in a carefully designed method-testing program, uncomplicated by variable performance of collaborative testers. However, the values should be confirmed by collaborative testing as will be discussed later.

Decision (*4*) is a matter of concern for any writer of methodology and must depend on external evidence. The writer (often a committee) may be too close to its work to judge its merits objectively. A procedure never can be described in sufficient detail so that it can be used by anyone. Rather the descriptive material is based on an assumed level of knowledge of a typical user. Any deficiencies in this respect could

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cause user problems. A collaborative test involving typical users obviously is a superior method for obtaining such evidence. It can demonstrate that the methodology, as described, can be used to obtain results in statistically significant agreement with the claims made for it.

Collaborative Test Rationale

The performance of a method should be evaluated using typical test samples. The selection of such is no problem for methods of limited applicability, although their suitability (e.g., homogeneity, stability, parameter level, etc.) may be complicating factors. For methods of wide applicability, a typical sample or a limited number of such will need to be selected, which may not be a simple matter. In either case, the cooperation of methodology experts with test-material experts may be required to develop and prepare suitable test samples.

The selection of a group of "typical" user laboratories may be influenced by such factors as willingness, availability of equipment, scheduling, public-spiritedness, and a feeling of urgency to get a method "in the books." Thus the sample may be more representative than the laboratories that participate. The latter is almost never a random sample of laboratories so that the interpretation of any statistics generated by the collaborative test may be debatable.

The general operational characteristics of a method should be well established before it is considered for standardization and the procedure should be "ruggedness-tested" (5) before the collaborative test. A peer or reference laboratory should have evaluated the performance characteristics. The collaborative test should confirm them or assignable causes for group or single-laboratory deviations should be sought. The need for suitable corrective actions of a technical or rhetorical nature resulting from the testing experience should be considered.

For those rare occasions when the precision cannot be established beforehand, the pooled within-laboratory standard deviations of the participants is all that can be reported. Again, outlying results need to be eliminated but the reasons for such will need to be investigated and their significance considered. It is hazardous to accept the results of any test that produced unexplainable outlying data.

Report of Collaborative Test

The collaborative test is a confirmatory exercise. While the statistics of the test may be reported and this is often done, only those of the peer laboratory, when they are available, are considered to be appropriate for describing the methodology. Between-laboratory statistics are seldom based on a statistically valid sample, hence they have little predictive value and are most useful only for diagnostic purposes. A test of a method should be considered successful when all participants can obtain results comparable with those of the peer laboratory and when no unexplainable outlying results are obtained. If changes are necessitated because of outlying performance, the proponents of the methodology must decide what corrective actions are necessary and whether additional collaborative testing is required. This author recommends that any publication of collaborative test results should designate them as "statistics of a test of the method" and not as "statistical parameters of the method."

Bias

The bias factor in methodology is often misunderstood. A method may be inherently bias-free; yet for various reasons, a laboratory may obtain biased results any time the method is used. The causes of any problems encountered when testing any method should be investigated and categorized as either inherent in the methodology or as artifacts of the measurement exercise. Only the former should be reported as bias of the method but insertion of appropriate precautionary statements to minimize the latter should be considered as well.

Most methods consist of comparison of an unknown sample with a standard, the accuracy and appropriateness of which are prime considerations. It is generally tacitly assumed that standards can be prepared with an accuracy greater than is needed, but this may not be so, especially in trace or high-accuracy analysis. When standards cannot be prepared with high precision, the random fluctuations of standards, prepared at various times, result in biases for any measurement referenced to a particular one. Appropriateness is concerned largely with matrix match and can be a critical problem when matrix influences are significant. In some cases, it is virtually impossible to prepare a standard that matches the sample that is analyzed. Often, only the sample presented to the measuring instrument is matched (or simulated) and other aspects of the measurement procedure are assumed to have insignificant error or are calibrated separately.

Extraction and dissolution inefficiencies can be both method-inherent and application-related and can produce random fluctuations around a bias. Losses and contamination each can appear as biases which can compensate under some conditions. Failure to correct for or the inaccurate correction of critical influences such as temperature and pressure can cause apparent biases. Interferences may cause biased results and always need to be considered in practical analysis, but are less important in collaborative tests where sample composition should be known and hence their elimination or correction is simplified.

Statistical Significance

If enough measurements are made, statistically significant differences between individual determinations are likely to be encountered. The question of the physical significance of such differences is one that can be answered only on the basis of professional judgment. There are no absolute criteria with respect to precision and accuracy but judgments must be made on the basis of practical considerations.

Statistical Control

The importance of attaining statistical control before and maintaining it throughout a collaborative test cannot be overemphasized. Meaningful tests cannot involve a learning exercise but expertise must be acquired beforehand. No results of measurement can be considered as having any meaning at all until such control is demonstrated (8).

Collaborative tests should be so designed that the system attains statistical control as evidenced preferably by control charts (9). Only then should the test samples be analyzed. Thus the collaborative test measurements are only a small part of the work that must be done in a meaningful test of a method. Those who advocate a few measurements of a few samples are well meaning but they ignore the first requirement of measurement—demonstration of the attainment of statistical control (8).

Significance of Within and Between Variance

Everyone who makes measurements knows that repeatability is more precise than reproducibility. That is to say, measurements made in a short time-sequence will agree better than those made over extended intervals of time. In fact,

a second reading of a scale may have little repetitive value (10) other than to prevent a blunder. In elegant physical calibration measurements, the short-term (within day) and long-term (between day) standard deviations differ little if at all (C. Croarkin, National Bureau of Standards, private communication). However, in complex chemical measurements, the latter may exceed the former by as much as a factor of 1.5 to 2, yet not result from significant measurement problems. This is because some of the factors contributing significantly to variance are constant or may vary insignificantly during a short period of time.

In a specific laboratory, the short-term variance is useful when deciding how the precision may be improved by repeated measurements. The long-term variance is the precision indicator that should be used to calculate confidence limits which indicate expected differences should the same sample be resubmitted for analysis at some future date.

The most meaningful within-laboratory standard deviation is its long-term standard deviation but this is difficult to evaluate on the basis of a collaborative test. The between-laboratory standard deviation may not be an estimate of the long-term standard deviation of a method for several reasons. Its evaluation, based on variances of the values reported by a group of laboratories, represents laboratory biases that may not be statistically distributed, which hence have little if any predictive value. When the between-laboratory variance is small, it means merely that little if any between-laboratory bias occurred during the test sequence. On the other hand, if it is large, the proponents of the method should be concerned that either the method is defective or that some or all of the participants were not in statistical control at the time of the collaborative test.

Validation

Collaborative testing can be used to validate methodology for a specific use but its function in this respect must be understood. Such a validation merely demonstrates the capability of the method to provide useful data (within acceptable limits of uncertainty) but does not guarantee that any data obtained using the method at any other time are necessarily valid. The author addressed this subject in an earlier paper (11).

Significance of Precision and Accuracy Statements

The precision and accuracy statements ascribed to a test method as the result of either peer laboratory or collaborative testing should be interpreted with caution. Presumably they summarize a test and are only as good as the test itself. They may be influenced by the design of the test plan, the kind and number of participants, the prior experience with the test by the participants, the fidelity in following the test plan and test procedure, and the quality of the test materials.

The statements can provide "figure of merit" information so that intelligent decisions can be made on the suitability of a method for a specific purpose. They may assist users in setting the initial control limits of a laboratory and for comparing its performance with that of others. While marginally useful in decisions on possible disagreement of isolated results obtained by users, only sound statistical data should be used for such a purpose (hypothesis testing).

No laboratory should use the results of a collaborative test or the reported statistics of a method to support any claims for the reliability of its data when using the method. This must be based solely on the statistics of its own measurement process while in a state of statistical control.

It should be remembered that a standard method envisions a standard measurement situation. The extension of such a method to measurement situations other than that for which it was tested may need further validation by collaborative testing or by the analyst any time it is used (11).

Conclusion

Reliable measurement data depend on appropriate methodology, adequate calibration, and proper usage. The results of collaborative testing can only provide information that may be useful in decisions on the appropriateness of methodology in a specific application, but the use of tested (validated) methodology does not guarantee results within its stated capability. One has only to look at the results of individual participants of a collaborative test, such as those of laboratory 8 cited in reference 12, to confirm this statement. Accordingly, publishers of standard methods should be sure that statements of precision and accuracy contained in them are not misleading. The inclusion of a disclaimer in this regard may be needed in some cases, such as for those methods used for regulatory purposes.

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Evaluation of Collaborative Studies with Special Consideration of the Outlier Problem

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Practical examples are given of outlier removal considerations when analytical aspects override the decisions of classical statistical tests.

In the Federal Republic of Germany, collaborative studies for checking methods of analysis are performed and evaluated in conformity with the procedure laid down in "Official Collection of Methods of Sampling and Analysis of Foods, Tobaccos, Cosmetics, and Articles the Surface of which may Come in Contact with Foods." The procedure is based on ISO standard 5725 (1) and is described in detail in a Codex document (2).

According to this procedure, 5 values are usually required from each participating laboratory. These 5 values are tested for possible outliers by the Grubbs test (2) at the 95% level (2-tail) and if the critical value is exceeded, 3 additional measurements are to be made to ensure that at least 5 values are available after outlier removal for statistical evaluation or to dilute the effect of outliers. The laboratories are requested to report *all* values to the Executive Officer of the collaborative study.

Outlier Evaluation

In the evaluation of collaborative studies, one of the most essential problems is the decision to eliminate single values or a series of values. An unjustified elimination, for instance, results in a smaller dispersion estimate than would in fact be reached in practice; on the other hand, keeping real outliers results in too high a dispersion estimate. Therefore, the decision for elimination of method-specific outliers must not be influenced by personnel, equipment, or local conditions. Statistical tests are an auxiliary means for the detection of possible outliers. The decision as to whether such values are to be considered as outliers and ought to be eliminated, however, is a separate process.

During the final evaluation of all of the results from the collaborative study, the Grubbs tests for 1 and 2 outliers as well as the Dixon test, all at the 99% level, are applied for the detection of internal laboratory outlying values. However, it must be noted that when 3 or more of the values of the series of 5 are practically identical, these outlier tests will lead to a significant result even if the deviations of the remaining values are not practically important. The evaluation of some outlier problems that have been encountered in collaborative studies of bakery products may be demonstrated by some examples.

Extreme Values Within Laboratories

Example 1.—False indication of excessive deviation within a single laboratory.

The 5 values from a single laboratory examining biscuits for cholesterol by the GC method were as follows:

188.5*	192.5 192.7 192.9 193.0	mg/100 g

The value 188.5 is an outlier by both the Dixon and Grubbs tests applied to this set of data. Yet the standard deviation calculated from all 5 values is relatively small, 1.9 mg/100 g, and considerably smaller than that calculated from the corresponding value obtained from the entire group of 7 laboratories, 11.4 mg/100 g. In fact, the standard deviation from the laboratory with the flagged outlier is the smallest of all the participants. Therefore, this value, judged against all the data, is not an outlier and should not be removed. This example illustrates the principle that the Executive Officer, not the laboratories, should decide regarding outlier removal.

Example 2.—Multiple false indications of excessive deviations.

The 5 values from each of 3 laboratories examining butter cookies for starch by a polarimetric method were flagged as outliers by the Grubbs test for the 2 highest or 2 lowest values:

Laboratory 2:	49.1*	49.1*	50.2 50.2 50.2
Laboratory 7:	49.45*	49.45*	49.5 49.5 49.5
Laboratory 9:	50.3*	50.3*	50.4 50.4 50.4

The r-value ($r = 2 \times 2^{1/2} \times s_r$, where s_r is the average within-laboratory standard deviation) for all 10 laboratories participating in the study, with no values omitted was 0.80 (g/100 g); with the extreme values from laboratory 2 omitted, r from the 9 laboratories was 0.61. The dispersion from value to value within the other laboratories was distributed more uniformly, but over a greater range, than those shown for laboratories 7 and 9. Again, in fact, laboratories 7 and 9 have the smallest within-laboratory variabilities of the group. These outliers are characterized in the same way as those in Example 1. However, the extreme values of laboratory 2 were eliminated.

Outliers Exhibiting Excessive Variability

Another type of outlier is a series of measurements from a laboratory exhibiting an extremely high dispersion when compared to the corresponding dispersions from the other laboratories in the study. To detect this type of outlier, the within-laboratory variances are examined for homogeneity by the Bartlett test for large and small (but non-zero) variances, and by the Cochran test for large variances.

Example 3.—Enzymatic determination of lactose in butter cookies (10 participants; both Bartlett and Cochran tests positive).

Lab. No.	*Mean value, g/100 g*	*Std dev., g/100 g*
7	3.340	0.0112
6	3.168	0.0134
12	3.102	0.0192
1	3.272	0.0217
9	3.294	0.0261
11	3.194	0.0284
13	3.330	0.0474
4	3.218	0.0482
8	3.402	0.0497
3	3.178	0.1085**

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Laboratory 3 shows a strikingly high variance, whose elimination reduces the repeatability, r, from 0.131 to 0.093 and s_r from 0.046 to 0.033. Therefore, elimination seems reasonable. But discussion with participants revealed that other laboratories also obtained dispersions of this magnitude when applying this method to similar products. Therefore the value was retained.

Example 4.—Determination of fat in butter cookies by extraction with petroleum ether after hydrochloric acid digestion (12 participants; Bartlett test positive, Cochran test negative).

Lab. No.	*Mean value, g/100 g*	*Std dev., g/100 g*
11	8.431	0.0084
12	8.438	0.0192
5	8.476	0.0208
4	8.299	0.0253
7	8.150	0.0292
3	8.214	0.0297
9	8.482	0.0319
6	8.287	0.0644
13	7.992	0.0920
2	8.234	0.1128
8	8.060	0.1140
1	8.325	0.1165

The data exhibit a bimodal distribution of standard deviations—7 laboratories exhibited a relatively low variability between 0.008 and 0.032 and 5 laboratories showed a high variability between 0.064 and 0.117. This pattern suggests that a reduction in variability may be possible by an improved description of the method or by additional practice or training. However, a repeatability standard deviation of about 0.1% fat is the customarily expected within-laboratory variability for this type of method and this value meets the requirements of the analysis. Therefore no additional work is justified.

Outliers Exhibiting a Systematic Deviation

A third type of outlier is a large deviation of the mean by 1 laboratory (or more) when compared with the means of the other participating laboratories. The Kruskal-Wallis test and analysis of variance (ANOVA) are applied to this case. If significant results are obtained, the potential cause should be sought to determine if the outlying data are the result of an avoidable systematic error, which might arise from the use of different equipment or from a personal interpretation of a color change, or from unavoidable systematic deviations.

Example 5.—Determination of orotic acid in butter cookies (photometric measurement after bromination; 11 participants). Laboratory 2 has a mean value 4 times higher than those of the others and can be removed on the basis of any statistical outlier test (Figure 1). An examination of possible causes revealed insufficient experience by this laboratory.

Example 6.—Determination of chloride in butter cookies (titration with $Hg(NO_3)_2$; 8 participants). Laboratories 1 and 8 provided all the results above 0.94 g/100 g (Figure 2). The reproducibility ($R = 2 \times 2^{1/2} \times s_R$, where s_R is the between-laboratory standard deviation) calculated from all the data is 0.127; with elimination of these 2 laboratories, R is 0.03. Although elimination of these 2 laboratories is justifiable on the basis of these results, the expert discussion resulted in a decision not to eliminate these values. It was felt that elimination would lead to an unrealistic value for reproducibility of this titrimetric method, whose performance requires considerable skill. The low value for R calculated without the indicated outliers cannot be achieved in actual practice.

Example 7.—Determination of moisture in butter cookies (12 participants). The distribution is clearly bimodal (Figure 3). A systematic factor such as humidity in the laboratory must be the cause, but clarification has not yet been obtained. The method was not adopted.

To summarize, statistical tests for outlying values and for significant deviations of the variances and mean values are

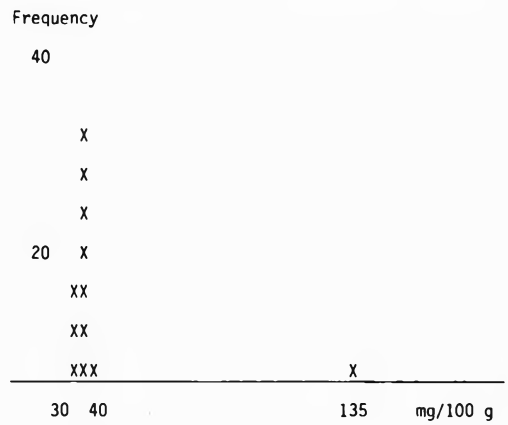

Figure 1. Frequency of results—Example 5.

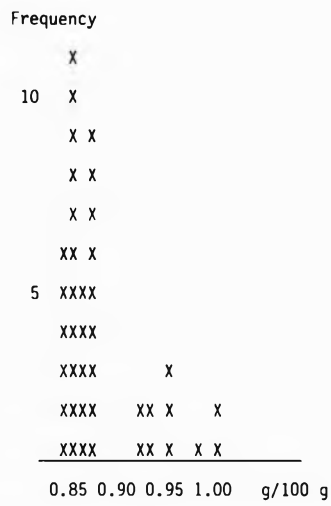

Figure 2. Frequency of results—Example 6.

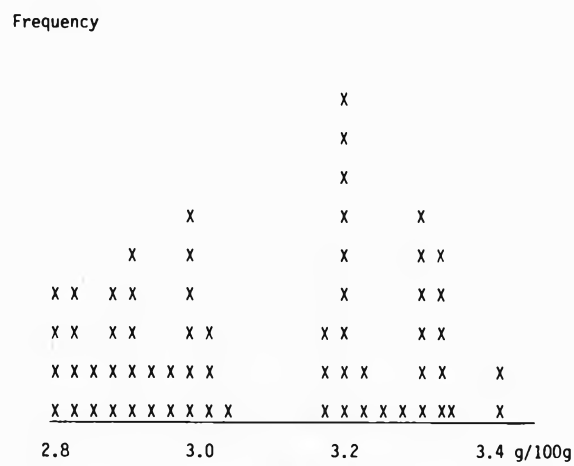

Figure 3. Frequency of results—Example 7.

necessary for the evaluation of collaborative studies. But the elimination of such "outliers" may not be done automatically. Rather it is essential to perform expert examinations to discover the causes, a task jointly to be tackled by the statistician and the analyst.

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Collaborative Testing of Methods for Food Analysis

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The complex composition of foods makes their analysis difficult. Results of collaborative tests with food materials often show greater coefficients of variation than with other matrices. Some critical points in collaborative testing of foods are discussed.

Like other biological materials, foods are difficult to analyze because of their complex composition. This usually necessitates a more or less complex procedure for separating the analyte. The concentration of the analyte in the original material is often low compared with the concentrations of interfering components. Furthermore, the analyte may be bound simultaneously in several chemical or physical ways, e.g., organic or inorganic mercury or tin; it may be present in several chemical forms, e.g., vitamin B_6; or it may represent a class or a group of substances, like fats, proteins, polychlorinated biphenyls, or toxaphenes. All these difficulties also constitute potential sources of error and consequently call for standardized methods of analysis, especially where the presence of the substance to be analyzed is regulated by law, statutes, or decrees.

The principle of collaborative testing, originally used only by some of the organizations developing or publishing standard methods for food analysis, has now been generally accepted. The procedure has developed over the years toward a more systematic approach to testing the characteristics of the method and to defining the criteria for accepting a method after the collaborative test. The principles and details have been concisely described by the AOAC Committee on Collaborative Interlaboratory Studies (1); their report contains detailed instructions for the referee to perform the collaborative test, and also lists the most critical points in the procedure. It is readily applicable to testing analytical methods for foods. In the following, some of the critical points and other experiences from collaborative tests in food analysis are presented.

Method Proposal

The quality characteristics of a method are mainly determined during the stage when the proposal for the method is prepared. It is essential that the selection of the principle and the procedures of the method is based on a thorough review of the literature; this should be followed by experimental work during which the most promising alternatives are compared, the selected alternative is optimized, and possible interferences are recognized. A commonly occurring pitfall is that the referee prefers a method he or she has earlier developed, modified, or used. Lack of optimization is often revealed in the interpretation of the results of a collaborative study, where the standard deviation is unnecessarily increased or the limits of application are diminished. On the other hand, a properly performed optimization provides clues for designing a collaborative test that will determine, with a larger experimental material, the effects of the critical factors or variables.

Collaborators

Selecting collaborators is a controversial task. A standard method should not be applicable only in the most advanced laboratories; all types of laboratories should be included. Often, however, the selection is limited by the availability of suitable equipment and previous experience in the techniques of the method. In any case, previous training in the techniques of the method with known samples is essential, and a collaborative test with too many beginners does not give a justified and reliable picture of the capabilities of the method.

Planning and Performing the Collaborative Study

Due to varying influences of the matrices, the samples are usually selected to represent the whole variety of foods for which the method is intended and the whole concentration range to be expected, unless some matrix or concentration is excluded on the basis of optimization experiments. Because of the various chemical forms and binding of the analyte, samples that have a known or independently analyzed inherent content of the analyte are to be preferred over spiked samples. However, spiking is often necessary to obtain data on recovery.

As an additional control of within-laboratory variability, the samples might include blind replicates or spiked levels that can be used to evaluate the correctness of the standards the collaborator is using. In some cases it is necessary to send material for calibrating instruments to all collaborators along with the samples.

To ensure homogeneity and good storage stability of the samples, a well minced, mixed, and dry or freeze-dried sample is the usual choice. Other possibilities are, e.g., chemically preserved, sterilized, or frozen food products in tightly closed containers. Samples of commercial food products originating from one well controlled production batch, or certified reference materials, may also be used. If samples have poor storage stability, collaborators are asked to per-

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form the analysis during a certain week, while the stability of the sample is tracked simultaneously by the referee. In some cases the only possibility is to send unknown samples of the analyte separately from the matrix, mixed in an inert medium, which is to be added by the collaborators to the matrices to be tested. This method is very seldom used in testing chemical methods, but sometimes has to be followed for microbiological methods. However, it introduces additional factors of uncertainty: differences in the matrices of the collaborators, the possibility of incomplete mixing, and the possibility that some collaborator may not mix the sample with any matrix.

Statistical Procedure

To ensure that all the desired characteristics of the method can be calculated, planning of the statistical treatment should begin when the program of the collaborative test is planned. To avoid mistakes, it is advisable at this stage to consult an expert in statistics. This planning should also include designing reporting forms, to obtain a correct number of original results from the collaborators.

After the results of the collaborators are received, a rapid first screening is advisable to correct obvious errors either in following the prescribed analytical procedure or in calculating or writing the results. In any case, it is important to find, in so far as possible, the reasons for outlying results, to be better able to evaluate the sensitive points of the method. This can best be done while the procedure is fresh in the memory of the collaborators.

Several statistical tests are available for finding outliers. The procedure presently used in our organization is ranking followed by Dixon's test, or alternatively we exclude values not falling within normal distribution. By calculating the standard deviations separately for each material and for each concentration range, one can evaluate the limits of applicability of the method.

The term "repeatability" is often interpreted to mean correspondence of results within one laboratory. However, in collaborative tests the results of each laboratory are usually obtained from only one operator on one occasion. This correspondence might be better called "replicability." The correspondence of the results within a single laboratory, achieved on different occasions by different operators, cannot be calculated from the results conventionally reported, unless this is included in the experimental design. Since there is also inconsistency in the definitions of the concepts "repeatability" and "reproducibility" and of the symbols used, a reconsideration of the terms, their definitions, and symbols is needed.

Statistical calculations should be made both for standard deviations and coefficients of variation. The latter can be used singly in the characterization of the method if the error is dependent on concentration. In other cases it should be used only when referring simultaneously to the concentration level or to the kind of sample.

Testing Microbiological Methods

Collaborative studies for testing microbiological methods have several drawbacks and limitations:

- The bacterial strain to be determined or identified as well as background flora is limited to those present in the samples to be sent.
- The bacterial flora may change from the time of dispatch to the time of analysis, unless the matrix and/or storing conditions prevent such changes. This limits the testing to organisms that are very resistant to conditions such as drying or freezing.
- To compensate for alterations in the bacterial flora, it is essential that all participating laboratories start the test simultaneously, which is seldom possible.
- For comparing substrates or cultivating conditions, 5 to 10 repeats of the tests are necessary to obtain sufficient experimental material.

For these reasons, comparative studies, in which each collaborator uses his or her own samples, have become the most common way of performing international testing of microbiological methods. To avoid differences in the composition of the nutrient media during the tests, it is often necessary to use substrates and reagents from the same batches, sent to the participants from the coordinating laboratory.

Interpretation and Conclusions

Compared with other materials, the results of collaborative tests with food materials often show greater coefficients of variation. However, expressed as a function of the concentration of the analyte, they usually fit within the limits generally achieved in AOAC tests (2). These limits can be regarded as guidelines, although not strictly binding, for evaluating the acceptability of a new method.

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View of the International Dairy Federation on Interlaboratory Analytical Studies

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The International Dairy Federation develops and studies methods for the analysis of milk and milk products. A first draft of an IDF protocol for conducting interlaboratory studies is presented. Major features of the protocol are type and number of participating laboratories; nature, duplication, and number of sample materials; final design (numbers of samples and laboratories); statistical analysis of the data; report of the final results.

1. Introduction

Looking at the numerous available documents dealing with interlaboratory studies, any organization, chemist, or expert who is not familiar with the subject and who wants to undertake such a study will inevitably be disoriented or even discouraged. From the basic and widely accepted design, consisting of identical materials sent to several laboratories which perform analyses with a given test method, several approaches concerning the organization and statistical evaluation, from the most simple to the most sophisticated, are possible. Two major documents usually have been referenced for designing an interlaboratory study: ISO standard 5725, "Determination of Repeatability and Reproducibility by Interlaboratory Tests" (1) and the Youden and Steiner *Statistical Manual of the AOAC* (2). Recently, the AOAC Committee on Collaborative Interlaboratory Studies produced "Guidelines for Interlaboratory Collaborative Study Procedure to Validate Characteristics of a Method of Analysis" (3), which is worth considering.

The International Dairy Federation (IDF) undertakes works in various scientific, technical, and economic fields and develops standard methods for the analysis of milk and milk products. Studies are done by groups of experts organized jointly under the aegis of IDF, the International Organization for Standardization (ISO), and AOAC, with the objective that each organization publish, in its own form and procedure, methods that are technically identical. Like many other organizations involved in the standardization of methods of analysis, IDF joint groups of experts conduct an interlaboratory study for any new or revised test method; now it is done systematically. Previously, no proper IDF standard was available and usually the ISO standard was used as a guide to conduct interlaboratory studies. This comprehensive document, however, is generally considered to be too extensive and too theoretical for practical guidance to IDF/ISO/AOAC groups of experts. The joint group E-30, Statistics of Analytical Data, has attempted to finalize a document based on ISO 5725 and the AOAC guidelines, which, it is hoped, will be helpful to the working groups.

2. IDF Needs and Specificity

Before discussing this document, it may be worth presenting some background information about IDF policy and philosophy concerning interlaboratory studies. It has been often emphasized that before an interlaboratory study is undertaken, its purpose should be clearly stated. An interlaboratory study may serve at least 4 different purposes. A study can be a standardization experiment, giving preliminary information about the various analytical attributes of one or more methods of analysis or helping in the preparation of the standard; it can be an experiment to determine the repeatability and reproducibility (precision) of the method; it can be used in laboratory quality control to check the performance of laboratories or analysts; and it can be used by agencies, like the National Bureau of Standards in the United States or the Community Bureau of Reference in Europe, to qualify standard materials.

Within the framework of IDF, the scope of interlaboratory studies with large international participation is restricted to the assessment of the precision of test methods. However, a laboratory study limited to members of the working groups is often used at first to compare and select methods according to their applicability and analytical attributes (precision, accuracy, limit of detection) and to check the adequacy and completeness of each step of the procedure. Finally, it can be said that when a method is submitted by IDF to an international interlaboratory study, it is usually meant that the method has already been recognized as a valid method by the group of experts.

Most IDF methods are reference methods which, by definition, are accurate, giving the accepted true value of the measured component; therefore, the protocol of the interlaboratory study can apply without restriction. At the previous symposium in Helsinki (4), we pointed out that the interlaboratory study procedure cannot be applied as such to indirect instrumental methods, except if standard reference materials are available that allow direct calibration of the method, or, as we will see later, if the method itself includes an accuracy test in its own procedure.

3. Major Features of the IDF Protocol

3.1. Laboratories

3.1.1. Type. Following the AOAC guidelines, we agree to stress that the participating laboratories must be impressed by the importance of the study because it may be the only such study that will be performed, and also because the precision figures that will be drawn from this study will be reported in the IDF standard and may, later on, be used as references by different countries. Contrary to AOAC standards, which are used nationally in the United States, IDF standards may or may not be accepted nationally as official methods for enforcement and regulatory purposes.

Concerning the participation of laboratories, and later the statistical analysis of results, the prevailing opinion is that selection of laboratories and rejection of results should aim to give the fairest estimate of the precision of the method so that the final figures are not biased by a lack of competence or practice from one or more collaborators. In that respect, the precision figures obtained from the interlaboratory study should be as close as possible to the values obtained in the pilot study conducted only with members of the working group who are supposed to be competent and familiar with the method.

3.1.2 Number. It has been well demonstrated, and everyone is convinced, that the main source of random error of an analysis is the variation of results among laboratories. There-

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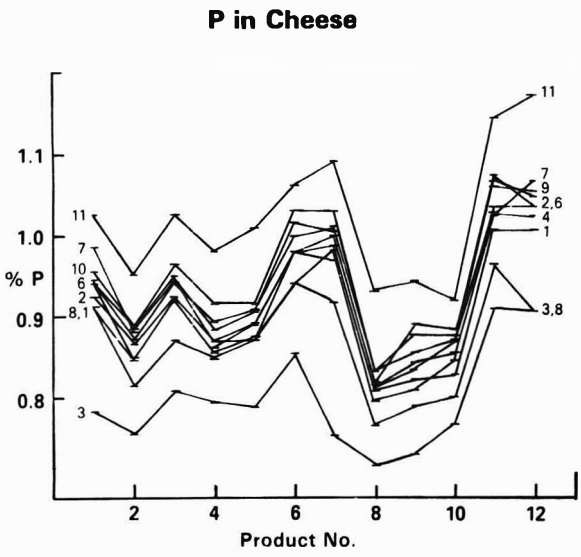

Figure 1. Phosphorus content in 12 samples of processed cheese analyzed by 10 laboratories by the molybdenum blue method (5).

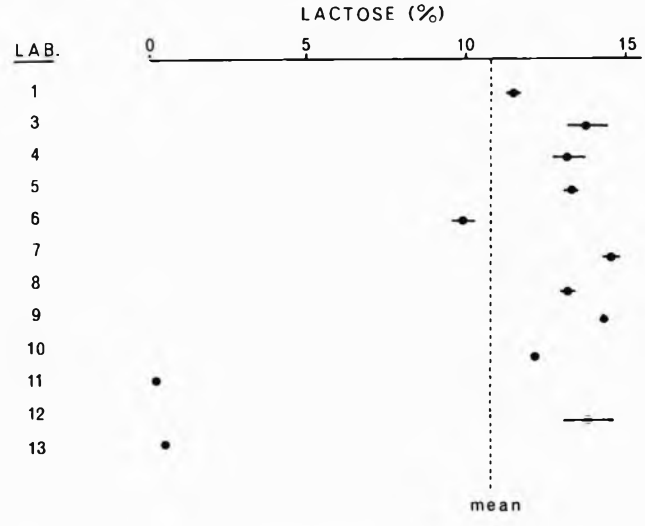

Figure 2. Lactose content (mean and range) of 1 sample of ice cream mix analyzed by 12 laboratories by the enzymatic method (IDF/ISO/AOAC Group E-6).

fore, a study that uses too few laboratories may lead to unreliable results. Systematic differences between laboratories stem from several sources: instrument calibration, reagents, accuracy of volumetric flasks, etc. In a recent study of the determination of phosphorus in processed cheese by the molybdenum blue method, Steiger et al. (5) showed that there is a significant laboratory-induced systematic error because of the lack of a suitable standard for calibrating the spectrophotometers (Figure 1). In this particular case, the presence of 2 real outlying laboratories was very helpful to demonstrate the need for an accuracy test in the procedure. Sometimes discrepancies between laboratories can also be related to the concentration of the analyte, but the magnitude of variation is not necessarily proportional to the level. For instance, testing the precision of the amido black method for milk protein determination, Grappin et al. (6) found that at the medium level (3.2%) the estimated laboratory readings differ by no more than 0.05% (one laboratory was excluded as clearly outlying), while at higher and lower protein levels agreement was much poorer, i.e., 0.10% at 2.6% protein and 0.18% at 4.0% protein. This indicates that instruments were calibrated against the reference Kjeldahl method only at the average protein level. This example illustrates the inadequacy of interlaboratory studies to estimate reproducibility when applied to indirect methods for which no standard reference material is available. In fact, a recent study conducted with 39 French laboratories has shown that when standard reference materials of different concentrations are used to calibrate the instruments, the standard deviation among laboratories decreases from 0.065% in the above mentioned study to 0.029%, and is nearly independent of the protein content.

To get an acceptable estimate of the standard deviation between laboratories and consequently of the reproducibility, and to improve the efficiency of the statistical test used for detecting outliers, participation in the study of at least 10 laboratories is highly recommended, with, it is hoped, no or very few outliers. However, it is clear that in certain circumstances this requirement cannot be fulfilled, simply because of cost or because there are only a few laboratories that are able to perform the analyses.

3.2. *Materials*

3.2.1. Nature. The field of application of IDF standard methods is generally narrower than for their AOAC or ISO counterparts, because most of the methods are product oriented, e.g., determination of nitrogen in liquid milk, phosphorus in cheese, nitrate in dried milk, or evaluation of the insolubility index of dried milks. Therefore, there are usually few choices as to the nature of the material used in an interlaboratory study. Even if a method is designed for the analysis of several products, like the enzymatic method for determination of lactose content, the field of application is always limited to dairy products.

It is important to point out the extreme diversity in the physical form of dairy products where we can find liquids with various degrees of homogeneity, stability, and viscosity; various forms like skim milk, cream, evaporated milk, butteroil, powder (milk or whey powder); and extremely heterogeneous products like cheese. This means that in many cases preparation of the materials for interlaboratory study will be critical and the requirement for homogeneous materials cannot always be easily fulfilled. In certain cases a unit-to-unit variability in composition is unavoidable and, therefore, this variability will be included in the precision of the method. Consequently, for a given method of analysis, slightly different precision figures can be obtained according to the material under test.

3.2.2. Duplicates. To simplify the design and the statistical analysis, the split-level or Youden pair design was deliberately omitted in the draft standard, and consequently laboratories will have to perform replicate determinations to estimate the repeatability of the test method. To limit the number of tests and to avoid unintended censoring of results, one determination per laboratory sample is required, using coded blind duplicates. However, one should not forget that the analyst will usually check his or her own repeatability by doing a replicate analysis from the same laboratory sample and not from blind duplicate samples.

3.2.3. Number. At least 3 different sample materials are necessary to comply with the minimum number of 30 values. Except when the normal variability of the analyte is low or if the method is especially meant for checking a specification level, the samples should cover the full range of variation of the component, with products at low, medium, and high levels. If we keep only 3 products, one at each level, the analysts, if aware of the presence of duplicates, will easily recognize them. To obviate this difficulty and lower the confidence interval of the estimates, 2 slightly different sample

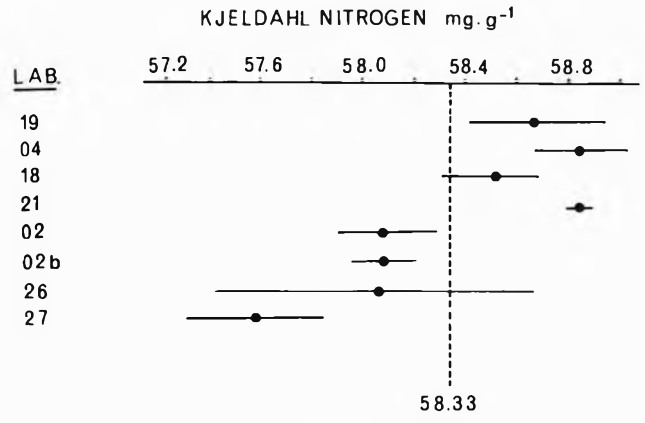

Figure 3. Kjeldahl nitrogen content (mean and range) of 1 sample of milk powder analyzed by 8 laboratories (8).

materials, instead of 1, are analyzed at each level, bringing the total number of materials to 6.

3.3. Final Design

Ultimately, the interlaboratory study will consist of 6 batches of materials, representing, if necessary, 3 different levels of concentration. Each material is split into 2 blind laboratory samples, which are sent to a minimum of 10 laboratories that are instructed to perform one test per laboratory sample. Subsequently, at the IDF/ISO Chemical Week in Rhenen, The Netherlands, May 6–10, 1985, the minimum number of laboratories was changed to 8, to agree with ISO 5725 (1).

3.4. Statistical Analysis

We mentioned earlier that one reason advocated for presenting a new document for interlaboratory study is the complexity of the statistical part of ISO Standard 5725 (1). Therefore, Group E-30 has strived to produce a simpler and more accessible document for nonstatisticians. However, if a standard that is too theoretical may discourage analysts, one that is too simple may lead to incorrect estimates and be rightly criticized.

3.4.1. Outliers. The first problem arising during the interpretation of collaborative data is the presence of outliers. Even if it is clearly stated that the purpose of the experiment is to check the method and not the laboratories, we should accept that the final precision value is greatly influenced by the ability of participating laboratories to correctly perform the method under study. As statistical tests, we simply recommend the Cochran test for testing within-laboratory variances and the Dixon test to check the mean differences between laboratories. Aberrant results are classified as outliers ($P \leq 0.01$) or as stragglers ($0.05 > P > 0.01$).

Data should be tested first for homogeneity of the within-laboratory variances (Cochran test). We are of the opinion that any laboratory found as an outlier with this test should be discarded, especially if it happens more than once. It is important to keep in mind that the final value must not be biased because questionable or unreliable results have been included. Second, laboratory means are tested for homogeneity. Before the Dixon test is applied, the means should be ranked and plotted. A "common sense" test based on a simple examination of the data points usually will give information about possible outliers. Before a laboratory is rejected on the basis of Dixon tests, results should be scrutinized; a rather conservative and critical attitude toward laboratories that are flagged outliers is advocated. Conversely, results from laboratories not found as outliers but that are located at the extremes of the distribution, especially if they have constantly low or high values for the different materials, also must be carefully examined before being accepted.

For instance, from the results given in Figure 2 (7), concerning a study conducted by IDF Group E-6 on the enzymatic determination of lactose in several dairy products, it is clear that laboratories 11 and 13 are outliers, but when the Dixon test is applied to laboratory 11, it is not found to be an outlier because of the masking effect of a second outlier in the same direction. Such a situation may occur each time the distribution of the population of laboratories is bimodal. In another study conducted by BCR (8) concerning the Kjeldahl nitrogen determination in one milk powder (Figure 3), no statistical outlier was found. But the results of at least one laboratory (Lab. 27) are questionable, because in this particular study the "true" nitrogen content of milk (58.8 vs 58.3 mg/g for the mean Kjeldahl) was given by the Dumas method. We know that the Kjeldahl method is prone to give low results because of the difficulty of achieving a correct mineralization of the nitrogen. Considering that the Dixon test is sometimes inappropriate to detect outliers, we would favor including a second test to be applied only when the working group considers that there are difficulties in the interpretation of the results.

3.4.2. Calculations. Precision values are calculated independently for each level or material by using simple formulas (limited to additions and squaring) drawn from a 1-way analysis of variance. If there is a single level, or if the standard deviations and/or coefficients of variation are identical at each level, the final figure is simply obtained by averaging the individual values.

3.5. Results

Besides the original data the final report should include the description of the method, the nature of the products or commodities analyzed, the number of laboratories included, the number of laboratories eliminated with the reasons, and the number of replicates. Precision parameters should be presented according to the model given in Table 1.

4. Conclusion

Presently, this IDF protocol for interlaboratory study has to be considered as a first draft. Following IDF rules, it will be circulated to the various IDF National Committees. Then it will be reconsidered by the group in the light of the comments received and also according to the final recommendations and conclusions of this symposium.

Table 1. Precision parameters obtained from an interlaboratory study

	Level (if necessary)		
Parameter	Low	Medium	High
Mean			
Repeatability:			
Standard deviation (s_r)			
Coefficient of variation (CV_r)			
Repeatability (r)			
Reproducibility:			
Standard deviation (s_R)			
Coefficient of variation (CV_R)			
Reproducibility (R)			
CV between-laboratories (CV_L)			

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Design and Conduct of Collaborative Studies: CIPAC Collaborative Experience in Pesticide Formulations

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CIPAC has set up procedures for carrying out interlaboratory studies on analysis of technical pesticide materials and formulations. CIPAC studies comprise several steps: allocation of leadership; method survey and selection; pre-collaborative trial; full collaborative study; evaluation of results and final decision on acceptance.

The main aims of the Collaborative International Pesticides Analytical Council Limited (CIPAC) are to promote international agreement on methods for the analysis of pesticide products and for the physico-chemical evaluation of technical pesticide materials and formulations and to foster interlaboratory collaborative analysis among interested laboratories.

Carrying out collaborative studies on a world-wide basis calls for a great deal of organization. To implement the above-mentioned intention and to direct the process, CIPAC has set up procedures for conducting interlaboratory studies. Although the real world situation may sometimes require modifications to and deviations from the accepted standard procedure, the basic principles remain intact. A short description of the main lines along which CIPAC collaborative studies are carried out is given below. The process is divided into several steps.

Step 1. Allocation of leadership for a particular pesticide.—Patented products are usually allocated to the CIPAC member of the country of the patent holder, and commodities are allocated to the member or country which has already done the main part of the analytical work or which otherwise wants to volunteer.

Step 2. Survey and choice of methods.—Method selection is a crucial point. The ultimate use of the method strongly influences the choice. Because CIPAC methods are intended to be used by regulatory and enforcement agencies as well as by laboratories dealing with certification, careful selection of a candidate method is important at this stage, and features like accuracy, precision (repeatability and reproducibility), specificity, and simplicity are emphasized. Much attention is also given to additional methods of identification.

Before the choice is made, methods described in the literature are surveyed, as well as methods available in members' and industrial laboratories. Such a survey often leads to useful information and contributes to rapid optimization of the method.

Step 3. Small-scale collaborative study.—The leader, having collected all needed information from the survey, can then draft an analytical procedure and check if it is workable. Before a collaborative trial involving many laboratories is organized, the workability of the method should be confirmed through testing by 2 or 3 other laboratories. Discussion among the few laboratories accelerates improvement of the description of the method, preventing errors and misinterpretation at the level of the full collaborative trial.

Step 4. Organization of the collaborative trial and recruitment of collaborators.—After the laboratories that have taken part in the small-scale study have agreed on the final version of the candidate method, the leader organizes a full-scale trial. Participation is not limited by invitation, but is open to anybody interested in the subject. Experience has shown that only very seldom do inexperienced laboratories take part. However, it may well be that some selection takes place as a consequence of the distribution pattern of the information letters sent by the CIPAC Secretariat. These letters, which include a short description of the method to be studied, the scope of the work, and the kind of apparatus and materials needed, are sent to CIPAC members, correspondents, and observers, to allied organizations, and to GIFAP (International Group of National Associations of Manufacturers of Agrochemical Products) which represents the pesticide chemical industry. This system of Information Sheets, which have a world-wide distribution, has proved to be an efficient tool for bringing together groups of interested laboratories.

Step 5. Report and acceptance of the method.—After the collaborators' results are received, the leader collates and analyzes them statistically, and prepares the report to be submitted at the annual CIPAC meeting. This report is then discussed and at the conclusion of the discussion, the decision is made either to accept the method as a CIPAC method or a provisional CIPAC method or to reject it. The predicate "provisional" is sometimes given to those methods for which the collaborative studies did not fully comply with the minimum requirements for number of samples and collaborators, but which otherwise complied with the basic principles of collaborative studies.

This last step actually consists of 2 steps, viz., the statistical treatment of the results of the collaborative study and the final judgment. To start with the first, any collaborative study would be useless without a planned study guided by statistics. CIPAC developed from a federation of national panels, so it is not surprising that different, parallel approaches exist. A synopsis of some studies carried out recently shows which statistical methods are being used (Table 1). The tests used cover nearly the whole spectrum of possibilities: the Youden method and the Steiner approach, as well as ISO standard 5725 together with its national variations. Under

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Table 1. Statistical treatment of some CIPAC collaborative studies

Statistic	Pesticide, method (country)						
	2,4-D, extn of acids (GB)	Diflubenzuron, LC (NL)	Maneb + fentin, titrimetric (GB)	Mevinphos, LC (D)	Nitrofen, GC (F)	Triazophos, LC (D)	Mecoprop, LC (DK)
No. of samples per study							
Technical products	2	3		1	1	1	2
Formulated products		3	4 + 2[a]	2	2	1	4
No. of laboratories	15	17	11	8	16	8	13
Replicates per study	2	blind duplicate	2	5	(2)–3	5	2
No. of results							
Technical products	2 × 30	51		40	39[b]	40	2 × 26
Formulated products		51	88 + 44	2 × 40	32[b] + 41[b]	40	4 × 26
Standard deviation	X	X	X	X	X	X	X
Coeff. of variation	X	X	X	X	X	X	X
Outlier evaluation							
Dixon test	X	X	X		X		X
Cochran test					X		X
Ranking test		X					X
Other				X		X	
Repeatability, r (95%)	X	X	X	X	X	X	X
Reproducibility, R (95%)	X	X	X	X	X	X	X
Statistical method reference	ISO 5725	Steiner	ISO 5725	Heinen-Ortner	NF 06041 DIN/ISO 5725	Heinen-Ortner	ISO 5725
Data reference	CIPAC Proc. 1981, p. 159	CIPAC Proc. 1981, p. 195	CIPAC Proc. 1981, p. 254	CIPAC Proc. 1981, p. 290	CIPAC Proc. 1981, p. 304	CIPAC Proc. 1981, p. 332	CIPAC Doc. No. 3113.R

[a]4 samples with fentin acetate, 2 samples with fentin hydroxide.
[b]After rejection of outliers.

CIPAC's direction this polyphony ends up in harmony. In all reports the important characteristics of the method, i.e., the repeatability and reproducibility, are calculated and severe violations of the principles underlying a collaborative study are absent. Recently, however, the call for more uniformity has grown. More uniformity would mean better and quicker comparison of the results of the various studies. However, some material may require special treatment so exceptions should remain a possibility. This symposium has clearly contributed in speeding up developments inside CIPAC.

One aspect not shown in the survey is the attention that is given to identification. During the study collaborators are asked to identify the compound(s) to be determined, e.g., by accurately measuring retention times or relative retention times. Additional identification methods such as IR spectroscopy, thin layer chromatography, etc., are sometimes given.

The last step, acceptance or rejection, is a difficult one, because no clear criteria exist as to when a method, assuming it has been studied according to the requirements, should be rejected. One way of solving this problem is to accept the empirical limits described by Horwitz et al. when they compared the variation of a few hundred collaborative studies.

It has been said that for a collaborative study to be valid it is required that the results be published in full. This certainly will stimulate the process of harmonization, giving people the opportunity to study the merits and disadvantages of other approaches. CIPAC can agree whole-heartedly with this suggestion. Numerous reports of CIPAC collaborative studies have been published in well known journals or in one of CIPAC's own publications, e.g., the Proceedings.

Role of IUPAC Commission on Analytical Nomenclature in Harmonization of Collaborative Analytical Studies

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The IUPAC Commission on Analytical Nomenclature (V. 3.) consists, at the moment, of 6 titular members, 12 associate members, and 8 national representatives. This membership is drawn from 14 different nations, representing the traditions and interests of western, socialist, and developing countries. With such a composition, the Commission is well suited to foster the aims of the Harmonization Symposium.

The importance of the Commission in the harmonization of collaborative analytical studies had already been recognized at the early organizing stages of the first Harmonization Symposium (Helsinki, 1981), when Harold Egan invited the then secretary (now chairman) of V. 3. to act as secretary to the organizing committee. It was the aim of the first Symposium to agree on at least the basic principles of collaborative studies; this could have been followed by nomenclature documents drafted by the Commission. Unfortunately, such an agreement was not achieved at the first Symposium, so the planned IUPAC contribution could not be realized. However, now, after the second Symposium (Washington, 1984), it is hoped that an agreement can be reached between the 2 seemingly different AOAC and ISO lines of thought. Once this happens, Commission V. 3. will be ready to prepare a nomenclature document. (At a Commission meeting held in Washington just after the second Harmonization Symposium, the matter was discussed at length. Members agreed that at the 33rd IUPAC General Assembly (Lyon, 1985) a new project on the "Nomenclature of Collaborative Analytical Studies" should be initiated under W. Horwitz (AOAC) as Coordinator with M. Parkany (ISO) and L. Currie (National Bureau of Standards), among others, as co-workers. These suggestions have to be endorsed by the various IUPAC authorities, but it is hoped that active work can start toward the end of 1985.)

Just after the 1981 Helsinki Symposium (as the result of some of the discussions there) at the 31st IUPAC General Assembly (Leuven), the Commission decided to revise the old nomenclature document on the "Presentation of Results in Chemical Analysis." This document has been finalized since then and copies were distributed to participants of the second Harmonization Symposium. These recommendations include new terms like "repeatability" and "reproducibility," the definition of geometric, harmonic, and quadratic means, and, as an entirely new section, definitions of quantities related to the use of linear calibration graphs. The latter topic formed a main part of my lecture at this Symposium, but will not be described here. The final document, in which account has been taken of the observations expressed at the Washington Symposium, will be published soon as a Provisional Report in *Pure and Applied Chemistry*.

As in the past, Commission V. 3. is looking forward to cooperation with subsequent Harmonization Symposia.

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[1]Chairman, IUPAC Commission V. 3. (1983–1985).

Analytical Quality Control in United Kingdom Water Industry, with Particular Reference to Harmonized Monitoring Scheme for River Water Quality

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The development of river water quality monitoring in the United Kingdom and the parallel development of analytical quality control (AQC) procedures within the UK water industry are described. Some results are presented for a sequential scheme of AQC which seeks to ensure comparability of analytical results obtained by different laboratories. The problems and advantages of such a scheme are examined, and future developments in nationally coordinated AQC in the water industry are discussed.

Before 1974, water services in England and Wales were provided by about 1400 separate bodies—river authorities, water boards and water companies, joint sewerage bodies, and local authorities. Only the larger organizations had comprehensive scientific departments, and the scope for detailed analytical quality control (AQC) to ensure comparability of analytical results on a national scale was severely limited. The emphasis was on obtaining operational data for day-to-day management purposes, although river authorities were involved in long-term surveillance work and reporting on the environmental state of their particular river systems.

In 1974 the government reorganized the UK water industry into 10 multifunctional Regional Water Authorities (RWAs) in England and Wales, dealing with water supply, sewage treatment, and river management.

In Scotland, Regional Councils deal with all aspects of sewage treatment and water supplies, while river basin management is carried out by River Purification Boards (RPBs). This paper will concentrate on the work of the RWAs and RPBs, but reference to water authorities will include private water companies where appropriate.

The scientific work of the water authorities includes the routine operation of sewage- and water-treatment works, statutory pollution control duties, and the management of rivers, estuaries, coastal waters, and groundwaters by the application of environmental quality objectives. The derivation and application of water quality criteria are required to protect public health, and to safeguard the consumer and the environment in general. The execution of these duties requires the provision of reliable analytical data to central government and various international agencies to fulfill European Community (EC) legislation and international conventions, such as the Paris Convention (which is concerned with the prevention of pollution of the sea from land-based sources). The needs of central government for water quality data have been defined by the Department of the Environment's Standing Technical Advisory Committee on Water Quality (1), which required the water authorities to supply water quality data to central government, to advise Parliament on matters concerning freshwater quality, to inform the general public, and to assist in national research and planning. The forthcoming implementation of the Control of Pollution Act will extend the requirement to include water quality data of estuaries and coastal waters. On an international basis, information has to be supplied to satisfy EC directives and other international commitments.

In 1972 the Department of the Environment (DOE), in conjunction with the then river authorities, developed a scheme to obtain quality and quantity data for freshwater rivers, and to achieve uniform standards of sampling and analysis. This was done to enable river water quality surveys to be carried out, to enable long-term trends in water quality to be identified, and to satisfy international obligations relating to substances carried down rivers into estuaries. A similar scheme was developed later to cover rivers in Scotland. Since the reorganization of the water industry, these schemes have been consolidated into a national Harmonised Monitoring Scheme for River Water Quality (the HM Scheme) and expanded to include additional determinands where information was needed in connection with UK international obligations. The full scheme is outlined in the Second Biennial Report of the Department of the Environment/National Water Council Standing Technical Advisory Committee on Water Quality (2).

Harmonised Monitoring Scheme

The objectives of the HM Scheme have recently been restated (1) following detailed consideration of the operation of the Scheme and possible ways to optimize the monitoring programs.

The restated objectives are as follows:

(1) To provide DOE with consistent, detailed information on concentrations of substances in water at representative points on the country's river system, sufficient to permit the identification of national trends in water quality.
(2) To meet the requirements for data on loads of materials entering estuaries from rivers.
(3) To meet the requirements of the EC Decision on the Exchange of Monitoring Information, and the WHO Global Environmental Monitoring Scheme.
(4) To remain sufficiently flexible to permit some adaptation to supply information which might be required both to meet future EC directives, and to support the UK position in negotiations on directives.

The first objective derives from the duty of the Secretary of State for the Environment to secure the effective execution of national policy that relates to the restoration and maintenance of the wholesomeness of rivers (Water Act 1973). The second objective arises largely from the need to satisfy international commitments, and the third and fourth objectives are self-explanatory.

Technical Aspects of Analytical Quality Control

The lack of comparable water quality data in 1974 gave added impetus to existing AQC work in the United Kingdom. In particular, the Water Research Centre (WRC) developed and promoted AQC procedures for use in the UK water industry (drawn together in a manual for the industry (3)),

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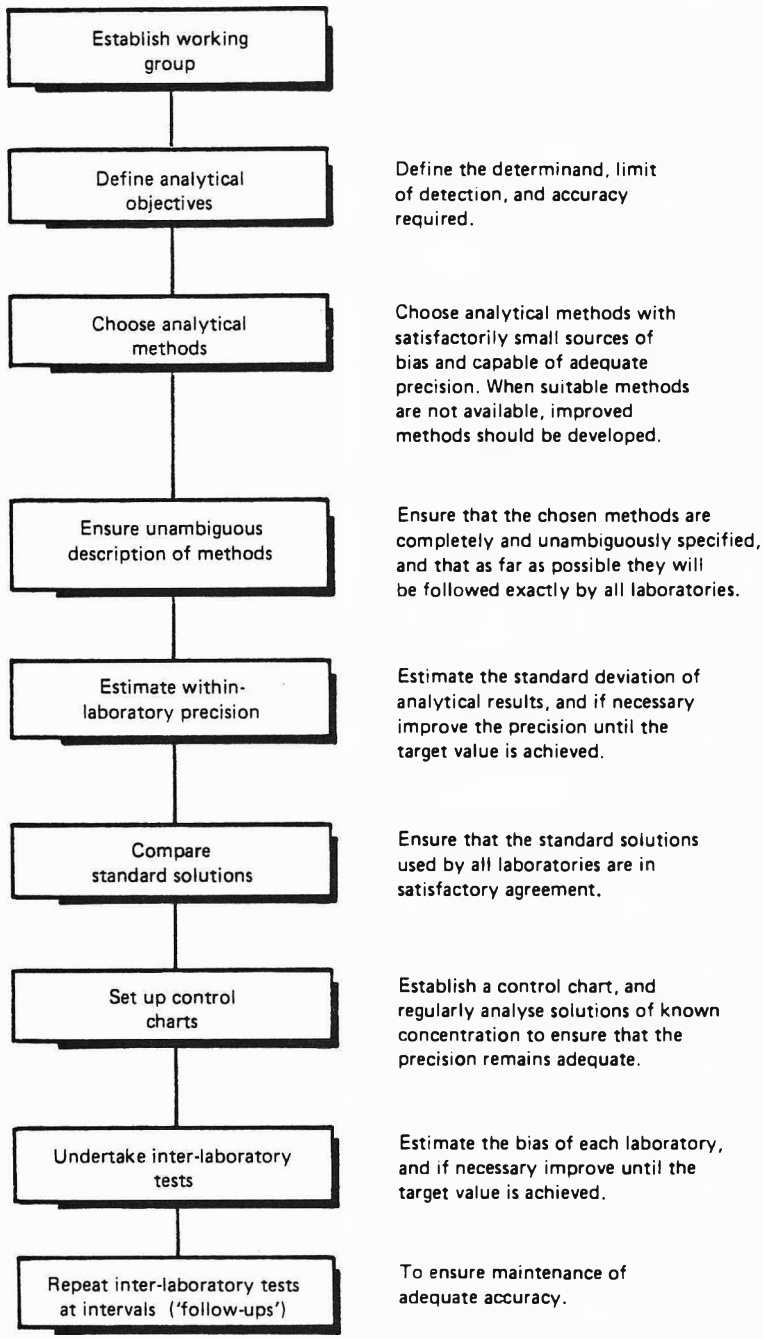

Figure 1. Flow chart for approach to achieving comparability of analytical results from a group of laboratories.

and DOE funded AQC work extensively through its incorporation of AQC in the Harmonised Monitoring Scheme described above.

Some of the concepts and procedures developed by WRC and the UK water industry formed the basis of our paper to the first IUPAC International Symposium on the Harmonization of Collaborative Analytical Studies, held in Helsinki in 1981 (4). In that paper we proposed alternative definitions for bias, precision, and criterion and limit of detection, and we presented a new definition for accuracy. (These definitions are reproduced in Appendix A.) We advanced arguments as to why our proposals were preferable to the definitions circulated by IUPAC, in advance of the meeting, to promote and encourage discussion in Helsinki.

Subsequently, we have pursued our definitions with the International Standards Organization (ISO) Technical Committee on Water Quality, via the British Standards Institution. We consider that our arguments were favorably received, although there was a majority view that the titles for our definitions should be changed as follows:

Accuracy changed to total error
Bias changed to systematic error
Precision changed to random error

These changes are acceptable to us. Our definitions for criterion of detection and limit of detection are still receiving consideration by the ISO Water Quality Technical Committee. However, it should be mentioned that ISO groups dealing with terminology have expressed some concern regarding our proposals, when compared with standard statistical terms in general technical use. We have stressed the application of our definitions to the problems of water analysis and the continued application of these concepts in the UK water industry further confirms the views expressed in our previous paper (4).

Table 1. Accuracy targets for determinands in Harmonised Monitoring Scheme, which have been subject to coordinated AQC[a]

Determinand	Precision target[b]	Bias target[c]
Chloride	5% of determinand concn or 0.25 mg/L	10% of determinand concn or 0.5 mg/L
Ammoniacal nitrogen; total oxidized nitrogen; nitrite	5% of determinand concn or 0.025 mg N/L	10% of determinand concn or 0.05 mg N/L
Suspended solids and ash	5% of determinand concn or 0.5 mg/L	10% of determinand concn or 1 mg/L
BOD (ATU)	0.25 mg O_2/L std dev. on sample measured in test (whether dilution or not). BOD of diluted samples to be >4.0 mg O_2/L on sample measured in test	0.5 mg O_2/L in sample measured in test
Pb; Cu; Ni; Zn	5% of determinand concn or 2.5 μg/L	10% of determinand concn or 5 μg/L
Cd	5% of determinand concn or 0.025 μg/L	10% of determinand concn or 0.05 μg/L
Hg	5% of determinand concn or 0.025 μg/L	10% of determinand concn or 0.05 μg/L
pH	0.05 pH unit	0.1 pH unit
Conductivity	5% of determinand concn or 1.25 μS/cm	10% of determinand concn or 2.5 μS/cm

[a]The larger of the 2 alternatives applies for any result.
[b]Target refers to total standard deviation of individual analytical results.
[c]Target refers to maximum tolerable bias.

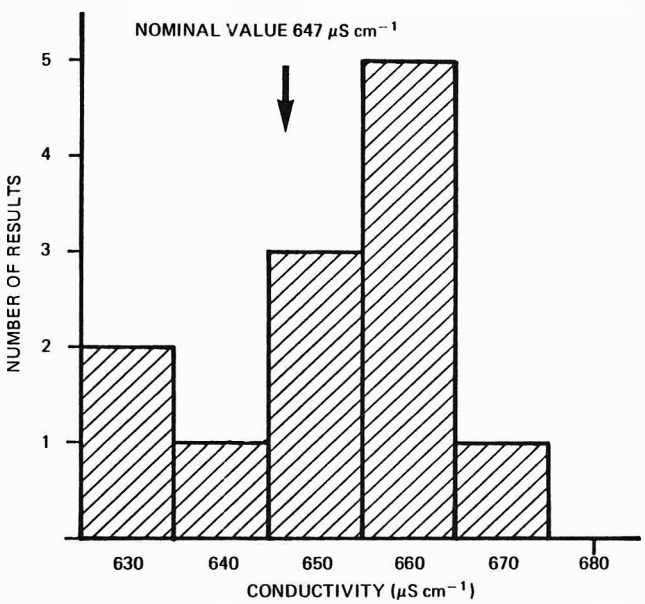

Figure 2. Results obtained in interlaboratory bias test for electrical conductivity.
Diagram shows histogram of mean results of different laboratories. Bias target was ± 10% of nominal value of potassium chloride solution, 647 μS cm^{-1}.

We also described a sequential scheme that had the objective of ensuring that comparable and adequately accurate analytical results were produced by a group of laboratories, in which the *interlaboratory test* was the *culmination* of a sequence of AQC activities. Figure 1 reproduces a flow chart indicating the steps required to achieve comparability of analytical results from a group of laboratories. Reference 5 gives fuller details.

This approach has been adopted in the United Kingdom in connection with monitoring programs for a number of different types of water. For example, it has been used to ensure comparability of results obtained by different laboratories engaged in a national survey of lead in tap water (6), and will shortly be used to ensure comparability of results for heavy metals in marine waters. However, its major application to date has been to ensure comparability of results obtained by laboratories engaged in the Harmonised Monitoring Scheme for River Water Quality, described above.

In connection with the HM Scheme, papers have been published describing application of this sequential AQC program to the following determinands: chloride (7), ammoniacal nitrogen (8), total oxidized nitrogen and nitrite (9), suspended solids (10), and electrical conductivity and pH (11). Reports on BOD (ATU), mercury, lead, copper, nickel, zinc, and cadmium are in press or in preparation. Accuracy targets for all these determinands are shown in Table 1.

The success of this approach to achieving comparability of results from a group of laboratories is illustrated in Figures 2 and 3, which show the results obtained by 11 laboratories in interlaboratory bias tests for electrical conductivity and pH, respectively (11) and in Figure 4, which shows the results obtained by 10 laboratories in an interlaboratory bias test for BOD (ATU) (12). It is noteworthy that, with respect to Figure 4, two laboratories did not complete all stages of the sequential AQC scheme shown in Figure 1 before undertaking the bias test, and the only failure to meet the bias target evident in Figure 4 was a result from one of those 2 laboratories.

For convenience, the AQC work for the HM Scheme is divided into 2 tiers. One laboratory from each organization or region participates in the first-tier stage, coordinated by WRC. After successful completion of the work, that laboratory then coordinates similar work for the second tier laboratories in its organization or region. Some 11 laboratories are involved in the first tier, and over 30 laboratories are involved in all. Essentially all first and second tier laboratories have completed the scheme in Figure 1 for chloride, ammonia, total oxidized nitrogen/nitrite, and suspended solids, but only at the first tier level is the work on the other determinands finished.

For chloride, ammonia, total oxidized nitrogen/nitrite, suspended solids, BOD (ATU), electrical conductivity, and pH, the AQC has been successful. The accuracy targets detailed in Table 1 have been met by virtually all laboratories which

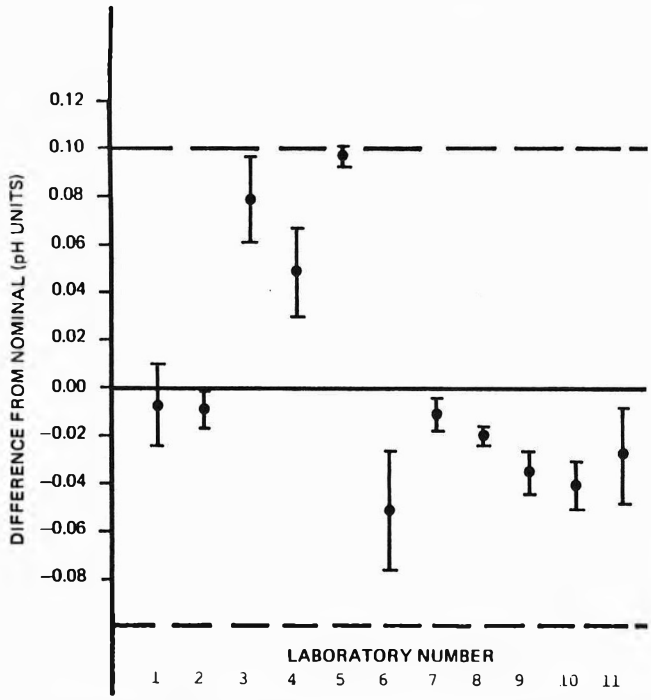

Figure 3. Results obtained in interlaboratory bias test for pH. Diagram shows mean deviation of each laboratory's results from nominal value of distributed buffer solution (pH 6.881) and its 90% confidence interval. Dashed lines are bias target values of ± 0.1 pH unit.

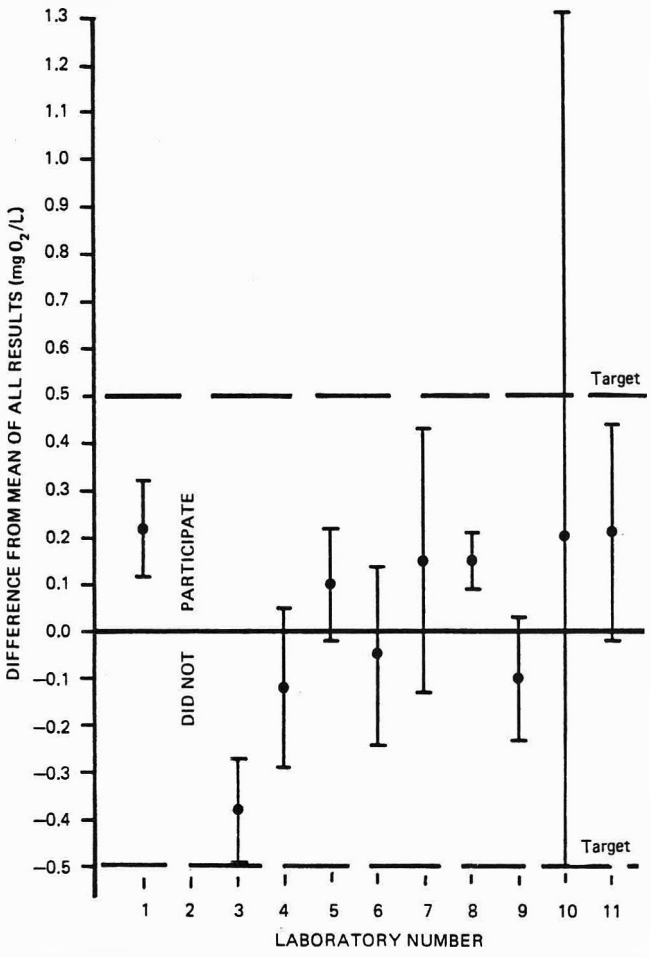

Figure 4. Results obtained in interlaboratory bias test for BOD (ATU). Mean of all laboratories results (3.82 mg O_2/L) for glucose/glutamic acid standard excludes results from laboratories 7 and 10, which did not complete entire sequential AQC scheme. Dashed lines indicate bias target of ± 0.5 mg O_2/L, and error bars indicate 90% confidence intervals on laboratories' mean results. Only one laboratory (10) failed to meet bias target.

have completed the stages shown in Figure 1, and this performance has been maintained subsequently over many years, as shown by the results of follow-up interlaboratory tests (see Figure 5). By contrast, it has not proved possible to achieve the required comparability of analytical results for trace metals from all laboratories, despite an *apparently* modest target accuracy of ± 20% for individual analytical results at the concentrations of major interest (see Figure 6). Possible reasons for this are discussed below; the success of the scheme shown in Figure 1 in achieving comparable results for lead in drinking water (4, 6) indicates that the problem does not lie with the AQC approach itself.

Problems Encountered in AQC Work

Progress in achieving comparability of results has been slow, especially for the trace metals (for which comparability within the specified targets has not been demonstrated). The main reasons for the slow progress identified by the group of analysts coordinating the work are (*1*) lack of well tested, proven analytical methods for trace metals at the start of the work; (*2*) limited effort available for AQC work in the participating laboratories exacerbated, in recent years, by the effects of industry reorganization.

The work of the Standing Committee of Analysts (4, 13) has since done much to rectify the lack of tested methods, but limited effort available for AQC work is likely to be a recurring problem in any activity of this kind. Lack of effort for AQC work, and the necessary method development/establishment stage in particular, has certainly hindered the water authorities' own coordination of the second-tier AQC work.

For cadmium, the original target accuracy (equivalent to the target limit of detection) applicable at low concentrations was 1 μg/L. This was found to represent an inadequate accuracy for the calculation of loads to sea, and a value of 0.1 μg/L has since been adopted. The difficulty of archiving results less than the limit of detection remains, however, and may have important consequences when loads are being calculated by multiplying river flows and determinand concentrations. If, as is usual practice, the analytical laboratory reports such results to the archive as "less than X," where X is the limit of detection, there is no means of knowing which value between zero and X should best be used in calculating loads or summary statistics (14). We recommend that the actual analytical result—positive, negative, or zero—be archived, together with an indication of the total error target, to avoid this problem. However, some analysts appear to have a rooted objection to reporting negative results, and the issue remains unresolved. Reporting the actual result should not, of course, be regarded as an alternative to adopting a lower target limit of detection but rather as a means of avoiding bias when the data are used subsequently.

Discussion and Conclusions

The operation of the Analytical Quality Control program for the Harmonised Monitoring Scheme has continued for a period of about 10 years, and has allowed an assessment of the advantages and problems of a coordinated AQC program to be made.

On the positive side, the required comparability of analytical results has been achieved for many determinands, and the desirability of, and approach to, interlaboratory AQC is now well appreciated throughout the participating organizations.

On the negative side, the required comparability has not been achieved for a number of important determinands and

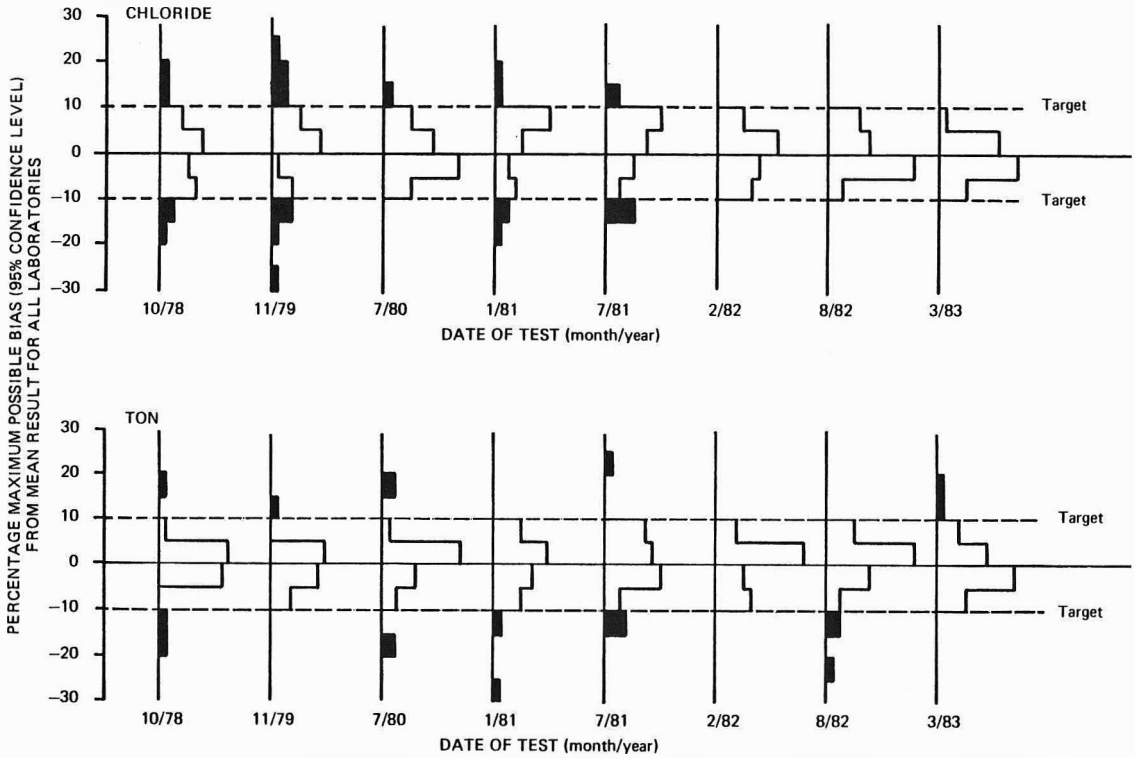

Figure 5. Results for follow-up interlaboratory bias tests for chloride and total oxidized nitrogen.
Top: Concentration range 15–40 mg/L. Between 22 and 27 laboratories participated in each test. Average of 86% of laboratories were within bias target. Bottom: Concentration range 3.5–8.7 mg N/L. Between 19 and 28 laboratories participated in each test. Average of 90% of laboratories were within bias target.

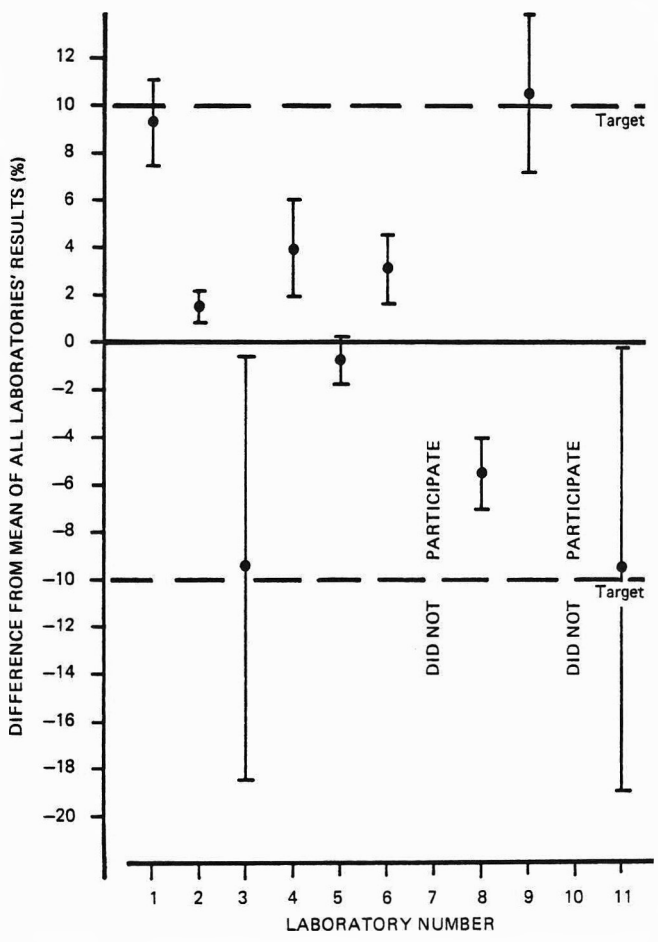

Figure 6. Results obtained in an interlaboratory bias test for total lead.
Distributed sample contained 51 μg/L of lead (mean result of all laboratories). Four laboratories (1, 3, 9, 11) failed to meet bias target. (Errors bars represent 90% confidence limits.)

the progress of AQC work has been slower than expected. The sequential approach shown in Figure 1 does require substantial effort from both the coordinating and participating laboratories, but the failure to achieve the required accuracy of analytical results for such "difficult" determinands as the trace metals suggests that a reduction in the rigor of the approach (e.g., by reducing the 10 day period over which the precision tests have been conducted) could not be undertaken lightly. It is considered that a major factor in the slow progress, and less than ideal outcome, of AQC work on the trace metals has been the lack of well tested, proven analytical methods at the start of the work. Another factor contributing to the difficulties experienced with the AQC work has been the reorganization of laboratories within the water industry, and the pressures on laboratory work and analytical effort—especially at a time of economic recession.

The current view of central government seems to be that the task of ensuring that analytical results are of adequate accuracy (and, therefore, comparability) is one which the UK water undertakings will need to perform for their own purposes; accordingly, DOE funding for the coordination of AQC for the Harmonised Monitoring Scheme will shortly cease.

It is certainly true that, in large measure because of the AQC work undertaken for the Harmonised Monitoring Scheme, funded by DOE, the scope and quality of AQC work throughout the UK water industry is now substantially greater than it was 10 to 15 years ago. It remains to be seen, however, whether the operation of such internal AQC programs will be sufficient to ensure adequate comparability of analytical results on a national scale. The difficulties experienced with trace metal determinations, even when nationally coordinated AQC was applied, suggest that the industry's internal AQC systems will require strengthening if there is to be any hope of success with such an approach. Moreover, it seems to us important that the effectiveness or otherwise of placing reliance on

internal AQC schemes for achieving national comparability be assessed by suitable interlaboratory tests, involving distribution of standards and/or samples as appropriate. Unless this is done, we believe there is serious danger of a lack of consistency in national data archives developing unnoticed.

It may also be observed that, if new analytical requirements arise as a result, for example, of further EC legislation, appropriate analytical methods will need to be developed and tested. The Standing Committee of Analysts has exercised this function for a number of years. As it completes its current program of method production, it is to be expected that areas of its work will close. However, there remains a need for a mechanism whereby analytical advances can be disseminated within the industry, and development and testing of new methods arranged.

The demise of nationally coordinated AQC work for the Harmonised Monitoring Scheme does not mean that all nationally coordinated AQC has ceased. WRC is currently commencing a program of AQC (following Figure 1) for the determination of mercury and cadmium in marine waters at very low levels (target limits of detection of 15 ng/L for mercury and 100 ng/L for cadmium). The majority of water authorities are involved. Again, WRC is also undertaking a short program of AQC work on pH determination in poorly buffered waters of low ionic strength, in connection with studies of surface water acidification.

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Permission of the United Kingdom Department of the Environment to reproduce Figures 2–6 is also gratefully acknowledged. The Analytical Quality Control work for the Department of the Environment's Harmonised Monitoring Scheme was funded by the Department.

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Appendix A. Proposed Definitions of Common Analytical Concepts

1. *Accuracy* denotes the total error of a result; that is, it represents the combined systematic and random errors. Accuracy is said to improve as the total error becomes smaller.
2. *Bias* is synonymous with systematic error. The mean of n analytical results on identical portions of a stable, homogeneous sample approaches a definite, limiting value, μ, as the number of results, n, is increased indefinitely. When μ differs from the true value, τ, results are said to be subject to bias of magnitude B, where

$$B = \mu - \tau$$

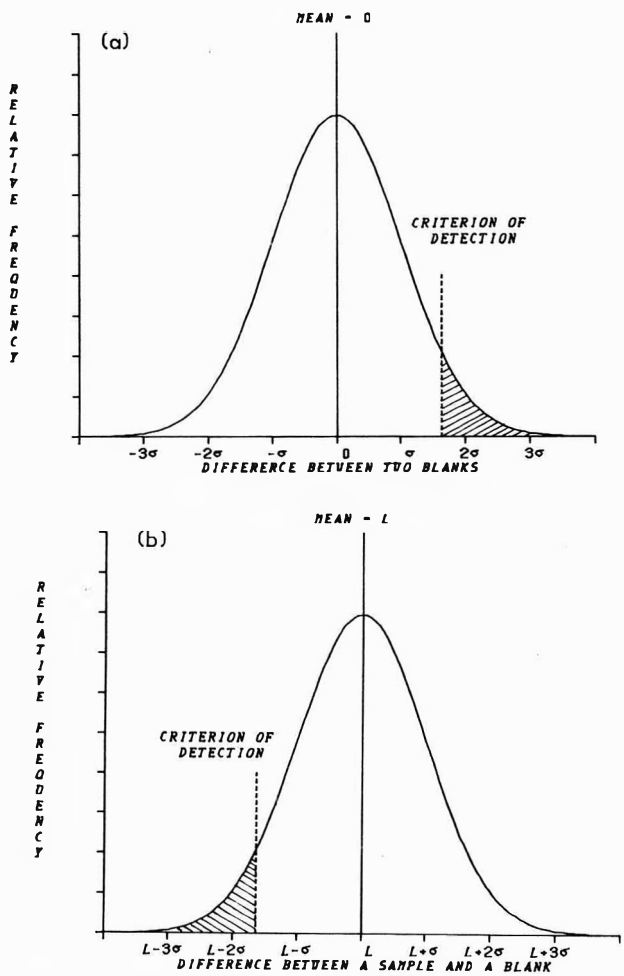

Figure A1. Statistical basis for detecting small concentrations. Shaded area denotes 5% of all results. (a) The difference between 2 blanks, and (b) the difference between a sample and a blank; L = limit of detection. ($\sigma' = \sqrt{2} \cdot \sigma_w$).

Because an indefinitely large number of determinations cannot be made on a single sample, the true value of μ (and hence of B) is, in general, unknown and only estimates of B will be available.

3. ***Precision*** is the closeness of agreement between the results of repeated analysis of identical portions of a stable, homogeneous sample. Precision is said to improve as the scatter among the results becomes smaller.

   The population parameter chosen to quantify precision (usually standard deviation) can, in general, only be obtained from an indefinitely large number of repeated analyses. For this reason, only estimates of standard deviation will be available.

4. ***Criterion and Limit of Detection*** (see also Figure A1). The *Criterion of Detection* is that concentration used by the analyst to judge whether the claim to have detected the determinand is justified. If a result greater than the criterion of detection is obtained, there is less than a certain probability (5% may often be considered appropriate) that the true concentration of determinand in that sample is actually zero.

   The *Limit of Detection* is the smallest concentration which the analyst can expect to detect with a given degree of confidence. (The 95% confidence level may often be appropriate.) Thus, if a sample has a determinand concentration just greater than the limit of detection, there is only a specified, small probability that a result obtained for that sample will be less than the criterion of detection.

   Both the criterion and limit of detection may be calculated statistically from a knowledge of the following:
   (*1*) the confidence level(s) required by the analyst,
   (*2*) the type of frequency distribution followed by results for samples of zero determinand concentration,
   (*3*) the standard deviation of the distribution (or, usually, an estimate of this parameter).

A Statistician's Approach to Repeatability and Reproducibility

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Repeatability and reproducibility are 2 concepts jointly describing the precision of tests performed according to some standard test method. The statistical principles underlying these concepts are explained in Part I. In Part II, these principles are applied to the design, statistical analysis, and interpretation of collaborative, or interlaboratory, studies especially organized for finding numerical estimates, s_r and s_R, of the repeatability and reproducibility standard deviations, σ_r and σ_R.

This paper is presented in 2 parts. Part I deals with the basic statistical principles, and Part II is concerned with collaborative studies, their design, analysis, and interpretation.

Some readers may be sufficiently familiar with the basic statistical theories and therefore may be interested only in the practical applications. They can skip directly to Part II. The numbering of the sections, tables, and references is continuous from Part I into Part II.

PART I

1. Tests, Test Results, and Standard Test Methods

In this paper a *test* will designate some measurement or determination—chemical, physical, or technological—that ends up as a single numerical *test result*. Whether that result consists of a single instrument reading or is obtained by combining a number of different readings is immaterial.

Tests are generally carried out according to a definite protocol, the *test method*. When that test method has been carefully validated and standardized by an organization such as AOAC or ISO (International Organization for Standardization), it becomes a *standard test method*. The purpose of standardization is to ensure that the same protocol will be applied in different laboratories so that the test results will be comparable. The following considerations are specifically concerned with standard test methods.

2. Variability in Repeated Test Results; the Statistical Model

Tests repeated under presumably identical conditions do not in general yield identical test results because it is impossible to keep all the factors that influence the outcome of the test under complete control. A *statistical model* serves to take this unavoidable variability in repeated tests into account.

That statistical model interprets every single test result as determined by a lot drawn at random from a population of items made up of all conceivable test results. The lots are drawn with replacement; that is, after reading the test result, the lot drawn is replaced in the population so that it has a chance of being drawn again at the next turn. "At random" means that at every draw all N items in the population have an equal probability, Pr = 1/N, of being drawn. Groups of items may be inscribed with identical test results, to account for the fact that some test results are more frequently observed than others.

3. Applicability of This Model

For this model to be applicable, a test method must satisfy 2 essential assumptions:

A1 The results of repeated tests must be in *statistical control;* i.e., the test method can be repeated indefinitely without change; and

A2 Repeated test results must be *mutually independent;* i.e., the outcome of any one of a series of repeated tests must not in any way be influenced by the other test results in the same series.

These conditions are a consequence of the rule that the lots must be drawn at random, without replacement.

Assumption A1 can never be fully satisfied, but within a limited set of replicates it can be accepted, because changes in the test procedure, if any at all, are too small to play a role. How far assumption A1 can serve for the interpretation of a set of test results has to be judged on the basis of a detailed knowledge of the circumstances under which the tests were repeated. We will return to this problem later.

This reservation also holds for assumption A2, although certain precautions may help to ensure its acceptability. There is always a danger that an operator, while repeating a test a number of times, will distrust the kth result when it differs too much from the previous (k − 1) results, and take some corrective action. In that case, the data would not be mutually

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Table 1. A set of 7 replicates with derived estimates and 2 ways of plotting

x_i = 5.23 4.34 4.03 4.66 6.66 4.21 4.31

Derived estimates:

Number of replicates = n,	n = 7
Mean = $\bar{x} = \Sigma x_i/n$,	$\bar{x}$ = 4.777
Variance = $s^2 = \Sigma (x_i - \bar{x})^2/(n-1)$,	s^2 = 0.842
Standard deviation = $s = (s^2)^{1/2}$,	s = 0.918
Coefficient of variation = $v = s/\bar{x}$,	v = 0.192 = 19.2%
Number of degrees of freedom associated with s^2 and s, $f = (n-1)$,	f = 6

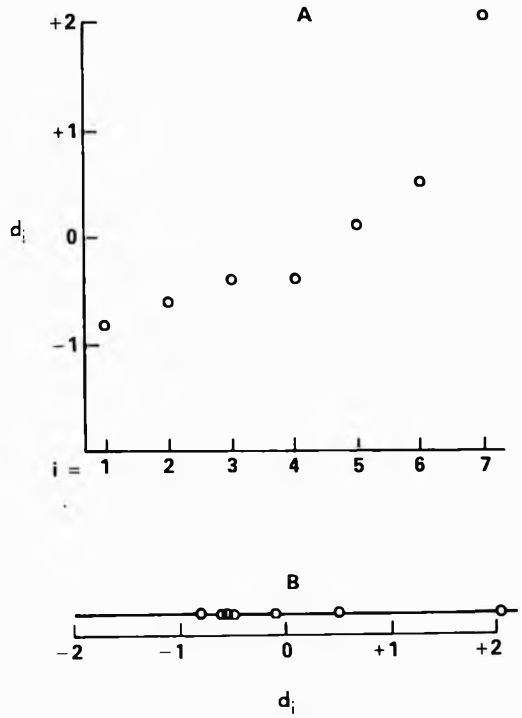
The test results rearranged in order of magnitude:

i =	1	2	3	4	5	6	7
x_i =	4.03	4.21	4.31	4.34	4.66	5.23	6.66

The deviates $d_i = (x_i - \bar{x})/s$ in order of magnitude:

d_i =	−0.81	−0.62	−0.51	−0.48	−0.13	+0.49	+2.05

The deviates plotted against their rank number (A) and on a linear scale (B)



independent. To prevent this, it is customary to prescribe that the tests must be carried out doubly blind. The operator must not know that he is repeating a test or must not know the test results before the series of replicates is complete. Assumption A2 is usually accepted as valid when such precautions have been taken.

In statistical theory such assumptions are presented as *hypotheses*. The consequences are then deduced presuming that these hypotheses are satisfied. In dealing with actual data we can never prove, but can sometimes test, whether these hypotheses are satisfied. To distinguish them from the technical tests of section 1, such tests of hypotheses will be designated as *statistical tests*. With a limited number of data, such statistical tests can only demonstrate comparatively large aberrations. Therefore, it seems more correct to say that in dealing with actual data we *assume statistical control and mutual independence*, and stick to these assumptions as long as some appropriate statistical test does not contradict them. What corrective action is needed if the assumptions do not seem to hold is one of the difficult problems of applied statistics, to which we shall return later.

4. Variates, Distributions, and Distribution Parameters

An observable quantity, x, for which the assumptions A1 and A2 hold, is known as a random or stochastic variable, or as a *variate*. We will use the latter term as the briefest.

A variate will be indicated by underlining the appropriate symbol. Thus $\underline{x}$ will be a variate, and x will be an observed or some specified value of that variate.

As explained in section 2, every variate is in theory associated with a population of all possible values, x_i, of that variate with corresponding probabilities, p_i, that these values will be observed. Jointly x_i, p_i define the *distribution* of the variate, and for practical purposes this distribution is chiefly specified by the following *distribution parameters:*

(1) The *mean*, μ (mu) or Expectation of $\underline{x}$,

$$\mu = E(\underline{x}) = \sum p_i x_i,$$

(2) The *variance*, σ^2 (sigma squared), or var($\underline{x}$), which is the Expectation of $(\underline{x} - \mu)^2$

$$\sigma^2 = E[(\underline{x} - \mu^2] = \sum p_i (x_i - \mu)^2,$$

(3) The *standard deviation*, σ (sigma), $(\sigma^2)^{1/2}$.

(4) The *coefficient of variation*, $V = \sigma/\mu$, or $100\,\sigma/\mu$ %. The symbols CV and RSD (relative standard deviation) are also used for this quantity.

The mean μ fixes the general level of the variate $\underline{x}$, while the standard deviation σ measures its variability. The variance σ^2 has, as we shall see further on, valuable theoretical properties and consequently plays a fundamental role in the analysis of more complicated experiments. In many cases the value of σ depends on the value of μ, whereas the coefficient of variation V is independent or nearly independent of μ. In such situations, the use of V is often preferred over the use of σ.

Variates can be discrete, taking only a discrete set of values as in the definitions (1) and (2) above, or continuous, varying on a continuous scale. In practice, continuous variables are handled as discrete variables because data have to be rounded to a limited number of decimals. There exist some differences in the mathematical treatment of the 2 types of variates, but these are not of interest here. The differences can be ignored, provided the rounding errors are small enough.

5. Distribution Function F(x)

Alternatively, the distribution of a variate can be represented by its *distribution function:*

(5) $$F(x) = Pr(\underline{x} \leq x),$$

which is the probability (Pr) that an observation of the variate $\underline{x}$ will turn out to be less than or equal to a specified value x.

If $\underline{x}$ is discrete, $Pr(\underline{x} \leq x) > Pr(\underline{x} < x)$ because the first probability includes the item $Pr(\underline{x} = x)$ and the second does not. Hence some care is required; for instance,

(6a) $$Pr(\underline{x} \leq x) + Pr(\underline{x} > x) = 1.00$$

Table 2. Percentage points of the standard normal distribution

P,% =	50	25	10	5	2.5	1.0	0.5
u_P =	0.000	0.675	1.282	1.645	1.960	2.326	2.576

$u_{(1-P)} = -u_P$

Table 3. Critical values for Grubbs' 2-tail outlier test

n	$\lvert d\rvert$ [a] 5%	$\lvert d\rvert$ [a] 1%	$s_{(n-1)}/s_n$ [b] 5%	$s_{(n-1)}/s_n$ [b] 1%
4	1.481	1.496	0.194	0.089
5	1.715	1.762	0.328	0.109
6	1.887	1.972	0.426	0.289
7	2.020	2.141	0.498	0.361
8	2.127	2.275	0.552	0.425
9	2.215	2.384	0.595	0.479
10	2.290	2.481	0.630	0.520
11	2.355	2.562	0.658	0.556
12	2.412	2.633	0.682	0.586
13	2.462	2.696	0.703	0.612
14	2.507	2.754	0.721	0.635
15	2.548	2.806	0.736	0.654
16	2.586	2.853	0.750	0.672
17	2.620	2.897	0.762	0.688
18	2.652	2.935	0.773	0.703
19	2.681	2.970	0.783	0.716
20	2.708	2.999	0.792	0.728

[a] $|d|_{max}$ = maximum value of $|(x_i - \bar{x})|/s$
[b] s_n = standard deviation before removing the suspected outlier; $f = (n - 1)$
$s_{(n-1)}$ = standard deviation after removing the suspected outlier $f = (n - 2)$

Note following relationship:

$$(n - 1)(n - 2)(s^2_{n-1}/s^2_n) = (n - 1)^2 - n(|d|_{max})^2$$

is correct for both discrete and continuous variates, whereas

(6b) $$\Pr(\underline{x} < x) + \Pr(\underline{x} > x) = 1.00$$

only holds for continuous variates because for these $\Pr(\underline{x} = x) = 0$. If the condition of the last sentence of section 4 is satisfied, the differences will be small, but it is always preferable to use formulas that are correct for both types of variates.

For a continuous variate, F(x) is a monotonic function of x, usually increasing with x; that is, $F(x_2) > F(x_1)$ if $x_2 > x_1$. A known distribution function can be tabulated by recording values of F(x) for given values of x, or alternatively by its so-called *percentage points* x_P defined by

(7) $$F(x_P) = P/100,$$

where the P's are specified probabilities expressed as percents.

These percentage points play an important role in statistical tests. We can, in addition to the basic assumptions A1 and A2 of section 3, introduce a hypothesis that completely specifies F(x) and its percentage points. For P = 99%, we have

$$\Pr(\underline{x} > x_{99}) = 0.01 = 1\%,$$

so that if our hypothesis is correct, values $x > x_{99}$ will be observed on the average only once per 100 test results. Hence when a single test yields an $x > x_{99}$, we must conclude that either a rare event has occurred, or else that the hypothesis from which x_{99} was derived is not acceptable. In statistics it is usually concluded that the observation $x > x_{99}$ is *significant at the 1% (or 99%) level,* and hence that the hypothesis *tested must be rejected;* in applications, a significant observation means that some further investigation is advisable. It may be that something went wrong with the data and that some observations should be rejected instead. What action is appropriate depends on the circumstances envisaged.

The argument applied above to an $x > x_{99}$ can, of course, also be applied to an $x < x_{01}$.

A great variety of statistical tests have been developed and are in use. Some examples will be discussed later. For the present our intent is only to explain the general principles.

Percentage points, such as x_{99} and x_{01}, are generally called the 99% or 1% *critical values* of the test.

At the present time, critical values corresponding to probabilities of 95% and 99% and/or 5% and 1%, are universally accepted as standard. This is the result of historical development; there is no theory that tells us whether one value is better than another. The choice is often a subjective one and has to be made in relation to the problem at hand. If, however, on the basis of a statistical test some decision of economic importance has to be taken, it is practical to have a standard set of values to adhere to.

An observation $x > x_{99}$ or $x < x_{01}$ is often called an *outlier* at the 99% or 1% level. Sometimes we are only interested in an upper outlier where $x > x_{95}$ or $x > x_{99}$ or in a lower outlier where $x < x_{05}$ or $x < x_{01}$ and then the test is a *1-tail test;* in other situations either an upper or a lower outlier may be of importance and we have to apply a 2-tail test. However,

(9) $$\Pr(\text{either } \underline{x} > x_{99} \text{ or } \underline{x} < x_{01}) = 0.02 = 2\%.$$

Hence, to keep the total probability of encountering an outlier at 1%, the critical values have to be changed to $x_{99.5}$ and $x_{0.5}$, and a similar change is required for the 5% level.

If Q and P > Q are 2 percentages, we have generally

(10) $$\Pr(x_Q < \underline{x} < x_P) = (P - Q)\%$$

and this relation is used for the construction of so-called confidence intervals (see section 12). In applying equation (10), it is customary to divide the total probability into equal parts over the 2 tails, i.e., to take Q = (1 − P). This again is an arbitrary but universally accepted standard procedure.

6. *Functions of Variates and Sums of Variates*

When $\underline{x}$ is a variate, any function $\underline{y} = y(\underline{x})$ will also be a variate, the parameters and distribution function of which are determined by those of $\underline{x}$. In particular we have

(11)
$$\underline{y} = a + b\underline{x} \text{ gives}$$
$$E(\underline{y}) = a + bE(\underline{x}), \text{ or}$$
$$\mu_{\underline{y}} = a + b\mu_{\underline{x}},$$
$$\text{var}(\underline{y}) = b^2\text{var}(\underline{x}), \text{ or}$$
$$\sigma_y^2 = b^2\sigma_x^2,$$
$$y_P = a + bx_P.$$

Hence,

(12) $$\text{if } \underline{u} = (\underline{x} - \mu)/\sigma, \text{ then } \mu_{\underline{u}} = 0 \text{ and } \sigma_{\underline{u}}^2 = 1.$$

Or: any variate can be converted by a linear transformation into a *standard variate,* i.e., a variate with $\mu = 0$ and $\sigma = 1$. Since linear transformations are mathematically easy to handle, standard variates form the basis of statistical theories.

In more complex situations we often have to consider a variate $\underline{x}$ as the sum of a set of different variates $\underline{x}_i$, each with its own mean μ_i and standard deviation σ_i. From

(13) $$\underline{x} = \sum \underline{x}_i, \quad i = 1, \ldots k,$$

it then follows that

(14) $$\mu = \sum \mu_i, \text{ or } E(\underline{x}) = \sum E(\underline{x}_i),$$

and if the $\underline{x}_i$ are all *mutually independent,*

(15) $$\sigma^2 = \sum \sigma_i^2, \text{ or } \text{var}(\underline{x}) = \sum \text{var}(\underline{x}_i).$$

The last equation explains the great value of variances in analyzing complex cases. The total error in test results can usually be interpreted as the sum of sub-errors due to different

error sources acting independently. By equation (15) and a suitable arrangement of our experiments, it is possible to find values for the different variance components, and thereby to judge how far and in what manner the error variance of the test method can be improved.

For instance, if there are 2 error sources and $\sigma_2 < \sigma_1/2$, equation (15) leads to

(16) $$\sigma < 1.12\ \sigma_1,$$

from which it follows that an improvement must be effected by reducing σ_1 in the first place.

7. *Applied Statistics: A Single Set of Replicates*

The results of a series of n tests, carried out under circumstances to which the assumptions A1 and A2 apply, will be designated as a set of n *replicates*. As an illustrative example, a set of 7 replicates have been reproduced in Table 1.

The distribution parameters of section 4 always remain unknown, but from a set of replicates we can derive a set of *estimates* of these parameters. These are

(17a) the mean $\bar{x}$ = an estimate of μ;

(17b) the variance s^2 = an estimate of σ^2;

(17c) the standard deviation $s = (s^2)^{1/2}$ = an estimate of σ

(17d) the coefficient of variation $v = s/\bar{x}$ = an estimate of $V = \sigma/\mu$.

The formulas by which these estimates are defined and the numerical values obtained are recorded in Table 1.

In addition, Table 1 contains the deviates $d_i = (x_i - \bar{x})/s$, for which $\bar{d} = 0$ and $s_d = 1$, which have been plotted in 2 different ways at the bottom of the table. By using such deviates, all series of n replicates can be transformed to the same scale, independent of the values of $\bar{x}$ and s, and thus intercompared. The plots present a visual picture of the distribution within the set of data and may be useful for spotting irregularities, such as outliers.

8. *Estimates as Variates; The Normal Distribution*

The next question is: "How good are our estimates?" By the assumptions of statistical control and mutual independence, sets of n replicates can be indefinitely repeated and estimates derived from repeated sets will be mutually independent. Hence these estimates can be seen as single observations of variates $\underline{\bar{x}}$, $\underline{s}^2$, $\underline{s}$, and $\underline{v}$; and the parameters of the corresponding distributions will determine what these estimates are worth.

The following formulas generally hold, independent of the specific distribution of $\underline{x}$:

(18a) $$E(\underline{\bar{x}}) = E(\underline{x}) = \mu,$$

which follows directly from (14) and (11);

(18b) $$\mathrm{var}(\underline{\bar{x}}) = \mathrm{var}(\underline{x})/n, \text{ or } \sigma_{\underline{\bar{x}}} = \sigma/n^{1/2},$$

which results from combining (15) and (11);

(18c) $$E(\underline{s}^2) = \sigma^2,$$

the proof of which must be omitted here.

By (18a) and (18c), $\underline{\bar{x}}$ and $\underline{s}^2$ are *unbiased* estimates of the corresponding parameters.

But these general relations do not suffice. In order to reach further conclusions we have to introduce a third assumption, namely,

A3 *The assumption of normality*, i.e., the assumption that the distribution of $\underline{x}$ can satisfactorily be approximated by the so-called *normal distribution*.

The chief properties of the normal distribution are as follows:

(19a) It is completely determined by the parameters μ and σ;

(19b) It is symmetric with respect to μ; i.e.,

$$\Pr(\underline{x} < (\mu - a)) = \Pr(\underline{x} > (\mu + a));$$

(19c) $\Pr(x)$ has a single maximum for $x = \mu$.

(19d) In theory, the normal distribution stretches from $-\infty$ to $+\infty$, but 99.73% of values fall in the range $\mu - 3\sigma$ to $\mu + 3\sigma$;

(19e) The standard normal distribution ($\mu = 0$, $\sigma = 1$) is completely fixed; its main percentage points are recorded in Table 2. From these we obtain the percentage points of any normal distribution by $x_P = \mu + u_P\sigma$. Henceforth, the symbols $\underline{u}$ and u will exclusively be used for a standard normal variate.

The assumption of normality, A3, automatically includes the assumption of statistical control, A1, but does not automatically imply mutual independence, A2. In view of equation (15), mutual independence is as important as normality.

As explained in section 5, the 5% and 1% points of certain distributions are universally accepted as critical values of statistical tests. As a rule, these percentage points are computed assuming normality and mutual independence, although the last condition is often incorrectly omitted. When the underlying distribution is not strictly normal, the result will be that the 5% and 1% values are only approximate. This is not serious as long as the statistical tests are commonly accepted as a basis for drawing conclusions or making decisions, because we do not know what percentages in any case are best.

9. *Testing Normality by Outlier Tests*

The common practice is that we accept the assumptions of normality and mutual independence as long as the data obtained do not in a certain sense contradict these assumptions by way of *normality tests*. With small numbers of replicates, deviations from normality manifest themselves primarily by the occurrence of so-called *outliers*, items in a set of replicates that lie rather far apart from the main body of the data. Hence in these cases, normality tests are usually carried out as *outlier tests*.

On the basis of past experience it may sometimes be possible by visual inspection of the data, or from such plots as presented at the bottom of Table 1, to decide that there is no outlier among them; or else that there is a *flagrant* outlier, the aberration being so large that any outlier test will lead to a significant result. But in case of doubt, an outlier test should always be applied.

Such a statistical test can only classify a suspected item as a *statistical* outlier. The next step is to investigate whether there may be some technical explanation—a wrong specimen was tested, an error in transcribing the data, etc.—in which case the outlier may either be corrected, or else may have to be rejected and, if possible, to be replaced by the result of a supplementary test. These are the *explainable* outliers.

It is the *unexplained* statistical outliers that are difficult to deal with. Experience has shown that they do occur in col-

laborative studies and have to be taken into account in analyzing the data.

The first problem is the choice of the level of significance. In keeping with general practice, outlier tests are generally applied with critical probabilities of 5% and 1%. But these probabilities refer to complete sets of n replicates for which the assumptions of normality and mutual independence hold true. In such cases, we will erroneously classify a valid item as a statistical outlier on the average only once in 20 sets of n replicates using the 5% level, and only once in 100 such sets using the 1% level. Hence from the viewpoint of the single test result these outlier tests are very conservative, the more so the larger the number of replicates in the set.

This conservatism has its disadvantages because the smaller the probability of classifying a valid test result as a statistical outlier, the greater will be the risk that a *real* outlier will not be classified as such.

Dixon (1) points out that the error introduced by rejecting a valid item as an outlier is generally less serious than the error resulting from a failure to recognize and reject a real outlier. From that point of view, a significance level of 10% for outlier tests should perhaps be preferable. Alternatively, it would be possible to change the critical values of the tests so that the probability of rejecting a valid test result would be independent of the number of replicates, but this has, as far as I know, never been proposed.

Table 4. Critical values for Dixon's 2-tail outlier test[a] (3)

Test criterion[b]	H	Critical values 5%	1%
$Q_{10} = \frac{z(2) - z(1)}{z(H) - z(1)}$ or $\frac{z(H) - z(H-1)}{z(H) - z(1)}$ whichever is the greater	3	0.970	0.994
	4	0.829	0.926
	5	0.710	0.821
	6	0.628	0.740
	7	0.569	0.680
$Q_{11} = \frac{z(2) - z(1)}{z(H-1) - z(1)}$ or $\frac{z(H) - z(H-1)}{z(H) - z(2)}$ whichever is the greater	8	0.608	0.717
	9	0.564	0.672
	10	0.530	0.635
	11	0.502	0.605
	12	0.479	0.579
$Q_{22} = \frac{z(3) - z(1)}{z(H-2) - z(1)}$ or $\frac{z(H) - z(H-2)}{z(H) - z(3)}$ whichever is the greater	13	0.611	0.697
	14	0.586	0.670
	15	0.565	0.647
	16	0.546	0.627
	17	0.529	0.610
	18	0.514	0.594
	19	0.501	0.580
	20	0.489	0.567
	21	0.478	0.555
	22	0.468	0.544
	23	0.459	0.535
	24	0.451	0.526
	25	0.443	0.517
	26	0.436	0.510
	27	0.429	0.502
	28	0.423	0.495
	29	0.417	0.489
	30	0.412	0.483
	31	0.407	0.477
	32	0.402	0.472
	33	0.397	0.467
	34	0.393	0.462
	35	0.388	0.458
	36	0.384	0.454
	37	0.381	0.450
	38	0.377	0.446
	39	0.374	0.442
	40	0.371	0.438

[a] This is R. S. Gardner's version of Dixon's test. This version applies when it is not known at which end of a series of data an outlier may occur.

[b] $z(h)$, $h = 1,2,\ldots,H$, is the series of data to be tested *arranged in order of magnitude*.

The most popular outlier tests are Grubbs' test based on deviates $d_i = (x_i - \bar{x})/s$, and Dixon's test using criteria of the general form

$$r_{ij} = (X_1 - X_{1+i})/(X_1 - X_{n-j}), \quad (20)$$

where the X_i are the test results arranged in order of magnitude.

Both tests can be applied as 1-tail and as 2-tail tests. For collaborative studies, the 2-tail tests seem to be most appropriate, as we do not know beforehand at which end of a series an outlier may occur.

In that case, Grubbs' criterion is $|d|_{max}$, the maximum value of the deviates taken in absolute value. An alternative, but equivalent, form of this test uses as a criterion the ratio s_{n-1}/s_n of the standard deviations derived from the full set of replicates, s_n, and after rejecting the most extreme item, s_{n-1}. The critical values for both cases are presented in Table 3. The second criterion illustrates the reduction in s achieved by rejecting the suspected item, and may be particularly useful from that point of view.

Dixon's 2-tail test uses as a criterion the highest of the 2 values obtained by applying (20) to the set of x_i when arranged in increasing and in decreasing order of magnitude, while the choice of i and j is made to depend on the size n of the series. A version used by AOAC (2) gave only the 5% critical values for a 1-tail test. Another version due to Crow, Davis, and Maxfield (3) is presented in Table 4, and is the one adopted in standard ISO 5725 (4) and more recently by AOAC (5). It differs slightly from the earlier AOAC version.

Applying these tests to the set of data in Table 1, we find

$$|d|_{max} = G_1 = 2.05, \text{ and}$$

$$s_{n-1}/s_n = s_5/s_6 = 0.428/0.918 = 0.466, \text{ and}$$

$$r_{10} = (6.66 - 5.23)/(6.66 - 4.03) = 0.544$$

By consulting the appropriate tables it is found that the test criterion lies between the 5% and 1% critical levels with Grubbs' 2-tail and Dixon's 1-tail test, but is not significant with Dixon's 2-tail test. Should the suspected item, 6.66, be rejected?

Other difficulties are encountered when there may be more than one outlier in a set. With Dixon's r_{10} criterion, a second outlier may prevent the detection of the first outlier, while with r_{22}, 2 outliers situated at opposite ends may be recognized simultaneously. If we apply both Grubbs' and Dixon's tests, or if we repeat a test after removal of an item, the 5% and 1% levels of probability are no longer strictly valid. This may not be a serious drawback because the choice of the 2 standard values is arbitrary, and we possess no other criterion by which to decide which is best, or whether some other level may be even better. Some criteria for testing the occurrence of 2 or more outliers have also been developed and may be worth considering (6). In frequently recurring situations, such as collaborative studies, it may be desirable to establish certain standard rules, regardless of the exact probability levels associated with them. An attempt in that direction has been made in ISO 5725, which uses only the Dixon test, permits repeating the test if more than one item is suspected, but only rejects an unexplained statistical outlier when it exceeds the 1% level.

So far this seems to have worked reasonably well in practice and to be an acceptable procedure in many cases. But it certainly cannot be considered as the final solution. As yet too little is known of the effect of rejecting outliers, and there is room for future research. All this illustrates that dealing with unexplained statistical outliers is a tricky problem, for

Table 5. Percentage points of the distribution of σ/s to be used for constructing the confidence interval for σ, given s and f

P,% =	0.5	2.5	25.0	75.0	97.5	99.5
f		a_1			a_2	
1	0.356	0.446	0.869	3.14	31.9	159.7
2	0.434	0.521	0.849	1.86	6.28	14.1
3	0.483	0.566	0.855	1.57	3.73	6.46
4	0.519	0.599	0.862	1.44	2.87	4.40
5	0.546	0.634	0.869	1.37	2.45	3.84
6	0.569	0.644	0.875	1.32	2.20	2.98
7	0.588	0.661	0.880	1.28	2.02	2.66
8	0.604	0.676	0.885	1.26	1.92	2.44
9	0.618	0.688	0.889	1.24	1.83	2.28
10	0.630	0.699	0.893	1.22	1.75	2.15
11	0.641	0.708	0.896	1.20	1.70	2.06
12	0.651	0.717	0.899	1.19	1.65	1.98
13	0.660	0.725	0.902	1.18	1.61	1.91
14	0.668	0.732	0.904	1.17	1.58	1.85
15	0.676	0.739	0.907	1.17	1.55	1.80
16	0.683	0.745	0.909	1.16	1.52	1.76
17	0.690	0.750	0.911	1.15	1.50	1.73
18	0.696	0.755	0.913	1.15	1.48	1.70
19	0.702	0.760	0.915	1.14	1.46	1.67
20	0.707	0.765	0.916	1.14	1.44	1.64

Example: for f = 10: Conf(0.699s $<\sigma<$ 1.75s) = 95%

$a_1 = a_2 = (f/\chi^2_Q)^{1/2}$, Q = 1 − (P/100)

which there is no hard and fast solution. Each case may have to be judged on its own merits.

10. s As an Estimate of σ; Confidence Intervals

Under normality and mutual independence, the ratios $\underline{s}/\sigma$ or $\sigma/\underline{s}$ will have distributions that depend only on f = (n − 1) = the number of degrees of freedom. For a first approximation, we have

(21) $\quad \text{var}(\underline{s}/\sigma) = 1/2f$, or $\sigma_{\underline{s}} = \sigma/(2f)^{1/2}$.

Percentage points of the ratio $\sigma/\underline{s}$ as recorded in Table 5 are used as follows: For n = 7, f = 6, Table 5 combined with equation (10) leads to

(22a) $\quad \Pr(0.64\,\underline{s} < \sigma < 2.20\,\underline{s}) = 95\%$;

and by filling in the actual value, s = 0.918 from Table 1, section 7,

(22b) $\quad$ Confidence $(0.591 < \sigma < 2.02) = 95\%$,

which is known as a 95% *confidence interval* for σ. The limits in (22a) are variates that will, for f = 6, include the unknown σ with a probability of 95%. In (22b) both the limits and σ are fixed but the uncertainty can still be considered to be expressed by 95% and is due to the fact that σ is and remains unknown. Hence statisticians prefer to call it a confidence instead of a probability of 95%. Confidence intervals in general, and 95% intervals in particular, are favorite statistical tools for expressing the precision of an estimate.

Table 5 has been designed for the construction of 1%, 5%, and 50% confidence intervals. It can be supplemented for other confidence levels as indicated at the bottom of the table. Sometimes only one of the confidence limits may be of importance; thus instead of (22) we may be content with Conf(0.591 $< \sigma$) = 97.5% or Conf($\sigma < 2.02$) = 97.5%.

A simple approximation to the 95% confidence interval is

(23) $\quad$ Confidence$[\{s/(1 + (2/f)^{1/2})\} < \sigma < \{s/(1 - (2/f)^{1/2})\}] = 95\%$,

which for f = 20 yields the multiplying factors 0.760 and 1.46 against 0.765 and 1.44 in Table 5.

From this last formula it can be deduced that to achieve a 95% confidence interval that estimates σ to within 20% requires a set of 60 replicates, and to achieve 10%, we need 4 times as many.

Such large numbers of replicates are, of course, practically never available, but we often have at our disposal a number of smaller series which can be considered as coming from variates all having the same value of σ. In that case, these can be combined into a single estimate s by

(24) $\quad s^2 = \sum(f_i s_i^2)/f$, with $f = \sum f_i$,

and in that way estimates of s with a reasonable precision can be obtained. Even from a series of k duplicates we can find in this way

(25) $\quad s^2 = \sum w_i^2/2k$, with f = k,

where the w_i are the differences between the 2 test results.

11. Coefficient of Variation

The coefficient of variation $v = s/\bar{x}$ is an estimate of $CV(\underline{x}) = V = \sigma/\mu$, where both $\underline{s}$ and $\underline{\bar{x}}$ are variates and their variability must be taken into account.

Under normality and mutual independence assumptions, $\underline{\bar{x}}$ and $\underline{s}$ are 2 mutually independent variates, regardless of whether $\underline{s}$ has been derived from the same set of replicates as $\underline{\bar{x}}$ or by some combination according to (24) or (25). For mutually independent variates, we have as a first approximation

(26) $\quad CV^2(\underline{s}/\underline{x}) = CV^2(\underline{s}) + CV^2(\underline{x}) = (1/2f) + (V^2/n)$.

From this we find an estimate

(27) $\quad CV^2(\underline{v}) = (1/2f) + (v^2/n)$.

In many situations, $v = s/\bar{x} < 0.1$ or 10%, and then the second term in (27) will be negligible compared to the first. This means that the factors in Table 5 can be used equally to establish a confidence interval for V, given v, as for σ given s. When both terms in (27) have to be taken into account, a confidence interval for V follows from the noncentral t-distribution, and is then of the general form:

(28) $\quad$ Conf$[(v/(1 + a_1\hat{CV}(v))) < V < (v/(1 - a_2\hat{CV}(v)))] = P\%$,

where the multipliers a_1 and a_2 are slightly different and are

functions of P and of the ratio of the 2 terms in (27) = $n/2fv^2$. For P = 95%, a_1 and a_2 lie between 1.90 and 2.00. When $a_2\hat{CV}(v)$ lies near 1, the upper limit in (28) will be very high and in that situation the use of the coefficient of variation is not recommended. This will occur only when v is high and n is small, a condition not often encountered in practice. Usually a confidence interval based on Table 5 will suffice.

Applying these principles to the data of Table 1, with v = 0.192, f = 6, Table 5 yields

(29a) $\quad \text{Conf}(0.124 < V < 0.422) = 95\%,$

while an exact computation according to (28) gives

(29b) $\quad \text{Conf}(0.116 < V < 0.598) = 95\%.$

The difference in the lower limit is unimportant, but that in the upper limit is quite noticeable. For this example, Table 5 is not adequate.

12. Mean $\bar{x}$ as an Estimate of μ

Under normality and mutual independence, $(\bar{x} - \mu)n^{1/2}/\sigma$ has a standard normal distribution, and s/σ has a distribution that only depends on the number of degrees of freedom, f, associated with s. Hence, the distribution of the ratio

$$t = (\bar{x} - \mu)n^{1/2}/s \tag{30}$$

is also determined by f. The t-distribution is symmetric with respect to zero, i.e., if Q = (1 − P), $t_Q = -t_P$. Therefore percentage points for P ≥ 50% suffice and are to be found in most text books on statistics.

From (30) we obtain by rearrangement

$$\Pr[(\bar{x} - t_P s/n^{1/2}) < \mu < (\bar{x} + t_P s/n^{1/2})] = 2P - 1, \tag{31}$$

with a corresponding confidence interval, given $\bar{x}$ and s.

For (2P − 1) = 95%, t_P varies around the value 2.0 and for a crude orientation it often suffices to use

$$\text{Conf}[(\bar{x} - 2s/n^{1/2}) < \mu < (\bar{x} + 2s/n^{1/2})] = 95\% \text{ approximately.} \tag{32}$$

The actual confidence levels of this approximation as a function of f are as follows:

f =	2	5	10	20	∞
Confidence level	81.6	89.8	92.7	94.1	95.5

It should also be emphasized that in (31) both $\bar{x}$ and s are variates, which implies that in confidence statements based on it we have to use each time a fresh set of estimates of $\bar{x}$ and s. If instead, we determine an estimate of s once and for all, and use it with different estimates of $\bar{x}$, the confidence levels may differ systematically from the intended level. It will be higher when s exceeds σ and lower when s is less than σ. This may not be too serious provided s is based on a sufficiently large number of degrees of freedom and we consider the confidence levels only as approximate.

PART II

13. Introduction

Standard test methods as described in Section 1 play an important part in our daily lives: in checking our health and our foods or in controlling the quality of all sorts of industrial products and materials. A correct interpretation of a set of test results has to take into account the possible effect of unavoidable errors in the data. It is consequently desirable to add to any standard test method some information concerning the magnitude of such errors. To provide this information is the essential purpose of collaborative studies or interlaboratory experiments discussed in this Part II.

14. Bias, Accuracy, Precision, Repeatability, and Reproducibility

Various error components must be clearly distinguished.

With some tests there exists a *true value*, x_T, the true concentration in chemical analyses, for example. In other cases the quantity tested may be defined by the test method so that there is no true value; the breaking strength of materials or the softening point of pitch may serve as illustrations.

When a test is repeated under conditions such that the statistical model of sections 2 and 4 can be applied, but the mean, μ, differs from the true value, the difference $(\mu - x_T)$ is known as the *bias* of the test method. The presence or absence of bias is generally related to the *accuracy* of the test method. A method is less accurate when it has a greater bias.

The variability among repeated test results, on the other hand, is interpreted as defining the *precision* of a test method. In keeping with the statistical model, the standard deviation, σ, is universally accepted as the most suitable numerical expression of that precision.

But this concept of precision has to be further subdivided because, as experience has shown, the value σ depends on the circumstances under which the tests have been repeated. The highest precision, or the smallest σ, can be expected under so-called *repeatability conditions*, i.e., among tests carried out simultaneously or in quick succession, in one laboratory, by one operator, using the same equipment throughout. We then have to deal with the *repeatability standard deviation*, σ_r.

The other extreme are tests repeated under *reproducibility conditions*, that is, tests carried out by the same method, but in different laboratories, because this will necessarily include variations in all possible factors that can contribute to the variability in test results. Often the *reproducibility standard deviation*, σ_R, is 2 to 3 times as large as σ_r.

Intermediate situations are also conceivable. Tests carried out within a single laboratory but on different days, or by different operators and/or different pieces of equipment, will correspond with standard deviations lying somewhere between σ_r and σ_R. If such intermediate values are needed, the conditions under which they apply should be specifically defined and investigated. The present discussion will be confined to σ_r and σ_R, which often suffice to deal with practical cases.

As a rule, the mean level of the test results, μ, can vary, sometimes within very wide limits. In such cases, the values of these standard deviations may depend on the level. This is a possibility that has to be reckoned with.

In theory, we would like to know σ_r and σ_R as functions of the level, μ; in practice, we have to fall back on estimates of these parameters, designated as $\bar{x}$, s_r, and s_R. We have to be content with establishing s_r and s_R as functions of $\bar{x}$, either as a graph or by a table. This should be seen as one of the basic purposes of a collaborative study.

Practical applications of a test method often have to deal with a single level of the property tested, an estimate of which will be provided by the mean, $\bar{x}$, of a few tests in one laboratory. From the data provided by a collaborative study, we can then derive corresponding values of s_r and s_R needed to judge (a) what that mean is worth; and (b) what conclusions can or cannot be based on it.

15. Validation Experiments and Collaborative Studies

Validation experiments are collaborative in the sense that different laboratories collaborate, but they serve a different

purpose, namely, to standardize a specific test method. This may include comparing different variations in the test procedure, or in the matrix, or studying the influence of contaminants on the test results. *Collaborative studies* is the term commonly used for experiments meant to determine the precision of a test method when the validation has been completed and a standard has been established.

These 2 types of experiments should be clearly distinguished. Only a small number of laboratories cooperate in the validation process and the experiments can be very varied.

In collaborative studies a larger number of laboratories must be included. But the experiments can be organized in a standard pattern, and the resulting data can be analyzed and interpreted by a standard statistical analysis. For example, a significant interaction between the level of the test property and laboratories may be important in validation experiments as an indication that the test method can be further improved. But once standardized, such an interaction has to be accepted as a feature of the method, and the effect of an interaction will be automatically incorporated in the estimates of s_R resulting from a collaborative study.

Pertinent to these observations, it is to be noted that the organization and interpretation of collaborative studies is the subject of an international standard specially designed for that purpose: ISO 5725 (4).

16. Samples Tested and Conditions They Should Satisfy

A further point to be observed is that σ_r and σ_R are meant to express errors due to the test method proper. Usually, however, the samples tested are destroyed by the test, so that for replications different portions must be used. These have to be drawn from batches of material that have been carefully homogenized beforehand, in order to avoid overestimating the standard deviations due to a variability among the portions. The official definitions of the repeatability and reproducibility in ISO 3534 (7) state that the tests must be made on *identical* material. But complete homogeneity is not strictly needed. If σ_P denotes the standard deviation between portions, that between test results will be given by (15)

$$\sigma_t = (\sigma_P^2 + \sigma_r^2)^{1/2} \quad (33)$$

and when $\sigma_P < \sigma_r/4$ this yields $\sigma_t < 1.03\ \sigma_r$. An increase of that order is perfectly negligible compared to the statistical uncertainty in the estimate s_r.

Whether a batch of material can be considered sufficiently homogeneous has chiefly to be judged on the basis of technical arguments. To demonstrate homogeneity by experiment would require the use of an alternative test method much more precise than the one studied. With fluids, or powders consisting of a single component, there may be no difficulties, but with substances such as pitch or butter, homogenization may be a problem. With mixtures of powders segregation is a disturbing possibility one always has to reckon with. Solid materials such as rubber sheets or metal rods can not be homogenized at all and have to be treated as another problem.

17. Repeatability

Owing to differences between operators, pieces of equipment, climatic conditions, etc., the repeatability standard deviation σ_r will not have exactly the same value in all laboratories. Determining a suitable estimate s_r must therefore, in the first place, be seen as a task for each laboratory separately.

For that purpose a series of n replicates will yield:

$$\bar{x} = \sum x_i/n, \text{ and} \quad (34)$$

$$s_r^2 = \sum(x_i - \bar{x})^2/(n-1) = [\sum x_i^2 - (\sum x_i)^2/n]/(n-1). \quad (35)$$

The second expression of s_r^2 is usually preferred for computation in order to avoid numerical errors that may be due to rounding of $\bar{x}$.

Alternatively an estimate s_r^2 can be derived from a set of n duplicates: x_{1i}, x_{2i}, $i = 1 \ldots n$, by

$$s_r^2 = \sum(x_{1i} - x_{2i})^2/2n = \sum d_i^2/2n. \quad (36)$$

Since carrying out duplicate determinations is often a standard laboratory routine, this may be more practical. This estimate may be considered as associated with the common mean

$$\bar{\bar{x}} = \sum(x_{1i} + x_{2i})/2n \quad (37)$$

when the difference between the individual means, $\bar{x}_i = (x_{1i} + x_{2i})/2$ are not too large.

Within a single laboratory it may also be expedient to plot the differences, $d_i = (x_{1i} - x_{2i})$, on a control chart in order to check the stability of the test procedure in the course of time. Suitable techniques are described in text books on statistics and/or quality control.

A serious *warning* is imperative here. It has been observed, and confirmed by special experiments, that operators are inclined, consciously or unconsciously, to censor replicate test results so as to bring the data closer together. Consequently σ_r may be underestimated, sometimes by as much as 50%. Hence the tests should be so arranged that the operators are not aware of the fact that they are carrying out duplicates or replicates; for instance by using coded portions mixed at random in a series to which the same test is applied, or by having the test results automatically recorded, the operator seeing the results only after the series has been completed. Duplicates may be easier to handle in this way than a larger set of replicates, and may be preferred for that reason. Tests carried out in this way are referred to as *blind tests*.

It should be noted, however, that physical tests, weighing for instance, may have to be repeated on the same object, and in such cases other steps may be needed to ensure mutual independence of a set of replicates.

18. Reproducibility

If a collaborative study is meant only to obtain an estimate for the reproducibility, s_R^2, it will be sufficient to send portions from a homogenized batch to different laboratories with the request for a single test according to the standard method. From the resulting data s_R^2 is obtained by (35), replacing s_r by s_R.

Strictly, if this is to be an unbiased estimate, the set of laboratories should consist of a sample drawn at random out of the population of laboratories using the standard in question. With a freely available standard this population will not be clearly defined and one has to be content with a set of laboratories judged to be reasonably representative.

Another point is that the precision achieved generally increases with practice and the observations should not be entrusted to operators carrying out the test for the very first time or after a long time lapse since they last carried out the test. It may then be advisable to provide the laboratories with some practice samples with which to gain experience before the official tests are carried out.

Table 6. The result of a basic collaborative study

Lab.	x_1	x_2	$\bar{x}$	$x_1 - x_2 = d$
1	21.2	21.4	21.30	−0.2
2	21.4	21.6	21.50	−0.2
3	20.8	20.7	20.75	+0.1
4	21.9	21.6	21.75	+0.3
5	21.0	20.9	20.95	+0.1
6	20.9	20.4	20.65	+0.5
7	21.2	20.9	21.05	+0.3
8	22.0	21.1	21.55	+0.9
9	20.7	21.0	20.85	−0.3
10	20.9	21.3	21.10	−0.4
11	21.1	20.6	20.85	+0.5

$n = 11$, $\bar{\bar{x}} = 21.12$
$s_{\bar{x}}^2 = 0.1296$, $f = 10$,
$s_r^2 = \Sigma d^2/2n = 0.0836$, $f = 11$,
$s_R^2 = s_{\bar{x}}^2 + s_r^2/2 = 0.1714$.

19. Basic Collaborative Experiment

As a rule collaborative studies are meant to furnish information both on repeatability and reproducibility. The simplest experiment then consists in sending 2 coded portions of a homogeneous batch of material to each laboratory to be tested as blind duplicates. The outcome of such a study is presented in Table 6.

The data are those of Sample 1 in Table 2 of page 74 of the *Statistical Manual of the AOAC* (2), after decoding by adding 20.

The analysis is primarily concerned with the variances s^2, the standard deviations being derived from these by extracting the square root:

$$s_{\bar{x}} = 0.360, \quad s_r = 0.289, \quad s_R = 0.414.$$

Outliers can be searched for by applying Grubbs' and/or Dixon's test (section 9) to the set of means, $\bar{x}_i$, and to the set of differences, d_i. These differences must be used with their sign included and the duplicates must have been recorded in the order in which they were observed. The reader may verify for himself or herself there is no evidence of outliers in the data of Table 6.

20. Youden or Split-Plot Experiment

In the basic experiment we use blind duplicates, as explained in section 17, which requires some special precautions. To avoid these, Youden (2) proposed duplicate tests performed on portions drawn from 2 different batches of material with slightly different levels of the test property. The operators will then no longer be inclined to censor their data to achieve a closer agreement.

The analysis of the resulting data is the same as in the previous section, except for the computation of s_r^2 which now has to carried out by

$$(38) \quad s_r^2 = \sum(d_i - \bar{d})^2/2(n-1) = [\sum d_i^2 - (\sum d_i)^2/n]/2(n-1),$$

with $f = (n-1)$.

From a strict statistical point of view this is not an unbiased estimate of σ_r^2, but an estimate of

$$(39) \quad E(s_r^2) = \sigma_r^2 + \sigma_{LS}^2,$$

where σ_{LS}^2 is the so-called variance component due to the interaction between the laboratories and the 2 levels of the batches of material used. However, this component reduces to zero when these 2 levels coincide, and the silent assumption underlying a Youden experiment is that with a slight difference in these levels the interaction component will be too small to play a role. If the validity of this assumption is doubted, a single material with blind duplicates should be used.

Whether the extra work of having to prepare 2 different batches of material in Youden experiments outweighs the advantages of avoiding blind duplicates seems questionable and may depend on the situation envisaged.

As pointed out in section 17 we cannot expect σ_r^2 to have identically the same value in all laboratories. Hence an s_r^2 by (35) or (36) is an estimate of the mean value $\bar{\sigma}_r^2$ taken over the laboratories collaborating in the study. From that point of view, applying Grubbs' or Dixon's outlier test to the differences in Table 6 can be interpreted as testing whether the variances σ_r^2 lie close enough together to be jointly represented by a single estimate s_r^2. In statistical parlance we assume homogeneity of the repeatability variances σ_r^2 among the laboratories and test whether this hypothesis can be accepted or should be rejected. In practice we reject 1 or 2 test results instead, and thereupon accept the hypothesis.

Similarily, by applying Grubbs' or Dixon's test to the duplicate means we test the hypothesis that the differences between laboratories can be described by a normal distribution, and if necessary uphold this hypothesis by correcting or rejecting some of the data.

21. Complete Collaborative Study

In some situations we may have to deal with only a single material, e.g., milk, and then a single experiment according to sections 19 or 20 may suffice. Usually however, the level of the tests may vary within wide limits and a complete collaborative study will consist of a number of basic, or Youden, experiments with batches of materials covering the range of levels that may be encountered in practice. An example is presented in Table 7, again borrowed from the *Statistical Manual of the AOAC,* (2, p. 78). The data have been decoded and the materials ("Samples" in the Manual) rearranged in order of increasing levels of the test property.

The bottom rows in Table 7 show that both s_r and s_R increase with $\bar{\bar{x}}$, the final values being more than twice the initial ones. This raises an important question that has not so far been clearly recognized.

The 3 estimates s_r in Table 7 can be considered mutually independent, being derived from independent sets of duplicates. But this does not hold for the s_R's. These are partly made up from differences between laboratories and these

Table 7. A complete collaborative study, with 3 levels, 10 laboratories, and duplicate tests[a]

	Level					
Lab.	1		2		3	
1	12.7	12.9	16.0	16.0	21.2	21.4
2	13.2	13.0	16.1	15.8	21.4	21.6
3	13.1	12.8	16.3	16.0	20.8	20.7
5	12.9	13.0	16.5	16.4	21.0	20.9
6	12.8	12.7	16.5	16.2	20.9	20.4
7	12.8	12.7	16.7	16.7	21.2	20.9
8	13.0	12.9	16.6	16.9	22.0	21.1
9	12.6	12.9	16.3	16.5	20.7	21.0
10	12.9	12.8	16.5	16.7	20.9	21.3
11	13.0	12.8	16.5	16.2	21.1	20.6
n	10		10		10	
$\bar{\bar{x}}$	12.88		16.37		21.06	
s_r^2	0.0175		0.0274		0.0875	
s_R^2	0.0222		0.0886		0.1390	
s_r	0.132		0.164		0.296	
s_R	0.149		0.298		0.373	

[a]From The AOAC Manual (2), p. 78, after decoding and rearranging in the order of increasing means $\bar{x}$.

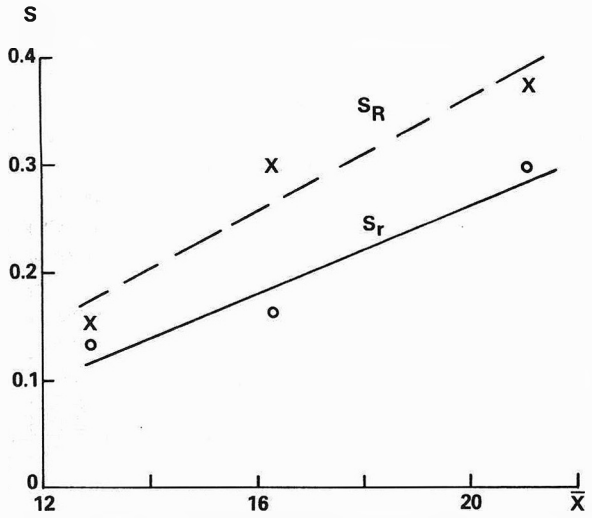


Figure 1. Measures of precision as a function of average values.

differences may be mutually dependent at the different levels. A possible consequence of this is that the s_R's may all 3 be infested with an error in the same direction, the magnitude of which it is difficult to assess.

From a statistical point of view it would be preferable to arrange a collaborative study so that the s_R's can also be considered as mutually independent. But to achieve this we would have to use a different and independent sample of laboratories for each level of the test property, which would greatly complicate the organization of the study and render it almost impossible. Hence the universally adopted procedure is to use one and the same set of laboratories for all levels.

Thereby, however, the pattern of the data becomes such that a 2-way analysis of variance (ANOVA) can be applied. This in turn has, in some quarters, led to the view that ANOVA is the correct way to analyze them. *This is a mistake!* The ANOVA carried out in the AOAC Manual leads to only 2 single estimates, viz.,

$$(40) \qquad s_r^2 = 0.0440 \text{ and } s_R^2 = 0.0832,$$

which are found to be the averages of the 3 distinct estimates in Table 7. Though the proof must be omitted here, it can be shown that this will always be the result. By a 2-way ANOVA we pool the variance estimates without looking at the distinct values for the separate levels and thereby miss seeing the functional relation between the variances, or standard deviations, and the level $\bar{\bar{x}}$.

To establish that relation the standard deviations have been plotted against $\bar{\bar{x}}$ in Figure 1. As by (21) the values of s_r and s_R may easily be infested with errors of 20% or even more; the 2 straight lines, adjusted by hand, satisfactorily fit the set of points. There seems to be no purpose in applying a more sophisticated fitting procedure. The resulting equations

$$(41a) \qquad s_r = -0.160 + 0.0216\,\bar{\bar{x}},$$

$$(41b) \qquad s_R = -0.204 + 0.0292\,\bar{\bar{x}},$$

sum up the chief results of this collaborative study.

It will be noted, however, that the use of more than 3 levels would be highly desirable, and that the expressions (41) are of very limited application; extrapolation to lower values of $\bar{\bar{x}}$ soon leads to negative standard deviations.

22. Some Further Notes Concerning the Example of Section 21

Originally 12 laboratories took part in the collaborative study of Table 7. Of these Laboratory 12 was rejected straight away because it had only carried out one test. Some other laboratories had produced 3 or 4 test results and these were reduced to duplicates by rejecting some items at random. These simplifications were necessary to carry out the ANOVA in a simple fashion.

If instead we carry out the analysis for each level separately, as in Table 7, unequal numbers of tests by different laboratories can be taken into account, though it complicates the calculations. The requisite formulas are to be found in the standard ISO 5725 (4).

Table 7 contains only tests in duplicate; it is equally possible to prescribe that the tests shall be carried out in n replicates, $n > 2$. In that case each combination of a level and a laboratory will produce a variance estimate $s^2_{(n-1)}$ with $f = n - 1$ degrees of freedom, and these have to be used in a variance homogeneity test. The test commonly used for this purpose is known as Cochran's test; a table with 5% and 1% critical values has been incorporated in ISO 5725. It is usually presented as a variance homogeneity test, but is in reality a 1-tail outlier test, as it uses the ratio of the largest s^2 over the sum $\sum s^2$ as the criterion. The smallest s^2 cannot effectively be tested because it is too strongly influenced by rounding of the data.

In other respects the analysis of a collaborative study with n replicate tests proceeds along the same lines as in section 21.

In the AOAC Manual, Youden has suggested that we should not only apply an outlier test to the data at each level, but also look out for outliers among the laboratories by testing them over all levels taken together. To that end the laboratories are ranked at each level according to their mean test results, $\bar{x}_i$, and their rank sums are used as the test criterion. For instance, in the original data from which Table 7 was derived, Laboratory 4 always had the highest mean, and consequently incurred a rank sum 33, significant at the 5% level. Hence all data for this laboratory were rejected, and are missing from Table 7. However, investigating this case a little further with outlier tests we find, as shown in Table 8:

Only at level 1 does Grubbs' G_1 come near the 5% critical value. Even if we add the data over all 3 levels, in the hope that thereby the effect may be enhanced, we still do not exceed that critical limit. Furthermore, the average reduction in s_R by rejecting Laboratory 4 is only 17%.

There are 2 other examples in the AOAC Manual (2, pp. 32 and 42) both with 5 levels and 10 laboratories, and each with 1 rank sum significant at 5%. In these cases the average reductions in s_R effected by rejecting the 2 culpable laboratories were only 10% and 6%.

23. Statistical Model and Meaning of the Statistical Tests

These observations lead inevitably to the conclusion that the rank sum test is too sensitive, and may, under some circumstances, unjustifiably reject all data from some laboratory. This raises the question: What statistical model lies at the back of this and other statistical tests, and what do these tests really mean?

Table 8. Should Laboratory 4 be rejected by the rank sum test?

Level	1	2	3	1+2+3	G_1 5%
Grubbs' G_1	2.287	1.683	1.755	2.337	2.355
s_{R10} with Lab. 4	0.200	0.335	0.414		
s_{R9} without Lab. 4	0.149	0.290	0.373		
s_{R9}/s_{R10}	0.745	0.866	0.901		

For a *single* level of the property tested it is assumed that a set of replicates within each laboratory is a random sample from a normal distribution with

(42) mean = m_j and repeatability variance = σ_{rj}^2,

the index j indicating the different laboratories. The variance homogeneity test then tests the hypothesis

(43) $$\sigma_{rj}^2 = \sigma_r^2,$$

that all variances are equal.

Non-significance is interpreted as proof that (43) is approximately correct, and that the variances can be represented by a common estimate s_r^2. A significant outcome of the test is usually due to either a single outlying datum or to an outlying variance, and it may be difficult to decide between these 2 explanations. An outlying variance may indicate that the technique of applying the test method is not up to standard in the responsible laboratory and should be improved. What action is needed will depend on the situation envisaged.

Grubbs' and Dixon's outlier tests applied to the replicate means $\bar{x}_j$ test the hypothesis that

(44) the $\bar{x}_j$ = a random sample from a normal distribution,

which implies the additional hypothesis that

(45) the m_j in (42) are a random sample from a normal distribution.

The variance

(46) $$\text{var}(\underline{m}) = \sigma_L^2$$

is known as the between-laboratory variance. The variance between single test results from different laboratories then becomes

(47) $\sigma_R^2 = \sigma_L^2 + \sigma_r^2$ = the reproducibility variance,

and between the means of n replicates

(48) $$\sigma_{\bar{x}}^2 = \sigma_L^2 + \sigma_r^2/n.$$

Combining (47) and (48) gives

(49) $$\sigma_R^2 = \sigma_{\bar{x}}^2 + [(n - 1)/n]\,\sigma_r^2,$$

and this formula was, for n = 2, applied in Table 6 to find s_R^2 from $s_{\bar{x}}^2$ and s_r^2.

The differences between the m_j's are due to systematic differences between the laboratories at the times the tests were carried out. Many different factors may contribute to these differences, and the assumption of normality of the m_j's is based on the general experience that under such circumstances an approximate normality usually results. Hence an outlier in the $\bar{x}_j$ is considered an indication that something went wrong and that some correction may be needed.

The rank sum test on the other hand serves quite a different purpose. The underlying hypothesis is that the m_j's at the different levels of the quantity tested are mutually independent. But this is not a logical proposition. The concept of reproducibility, as distinct from the repeatability, has sprouted from the general experience that even the most careful validation of a test method cannot completely eliminate systematic differences between laboratories. These must be reckoned with. It is quite conceivable that some systematic differences between the laboratories will have the same sign at all levels of the test property with one laboratory producing test results systematically higher than another. Indeed taking the 3 levels in Table 7 pairwise we found an average correlation coefficient between the duplicate means $\bar{x}_j$, $\bar{x}_j'$ of 0.57, which increased to 0.78 when the rejected Laboratory 4 was put back in its place. This shows that a high rank sum is coupled with a pronounced correlation between the data from different levels of the test property.

A further investigation of the collaborative studies in the AOAC Manual reveals: *(1)* that the rank sum test may reject items with the lowest (or highest) rank number that are not in the least significant by Grubbs' or Dixon's outlier test, and in addition *(2)* may reject items with the second or third lowest (or highest) rank number, causing a reduction in the estimate s_R of only a few percent, or sometimes no reduction at all.

Summarizing: There is no reason to expect that rejecting a laboratory on the basis of the rank sum test will lead to a real improvement in the estimates s_R. Such a rejection should never be practiced without first verifying that the reductions in the s_R's are worthwhile. It may be added that, should we adopt the better policy of using different groups of laboratories for the different levels (section 21), the rank sum test would not be applicable and data otherwise rejected by the rank sum test would then have to be accepted, unless rejected by an outlier test.

24. Using s_r and s_R in Practice

The difference $y = (x_1 - x_2)$ of 2 independent test results from the same normal distribution, (μ, σ_x), will be normally distributed with mean = zero and $\sigma_y = 2^{1/2}\,\sigma_x$. Consequently

(50) $$\Pr(|y| = |x_1 - x_2| < 2 \times 2^{1/2}\,\sigma_x) = 95.44\% \approx 95\%.$$

This has stimulated the definition of

(51) the repeatability interval = $r = 2 \times 2^{1/2}\,s_r$, and

(52) the reproducibility interval = $R = 2 \times 2^{1/2}\,s_R$,

with the added specification that these are values below which the absolute difference between 2 test results obtained on identical material by the same (standard) method in one laboratory (r), or in 2 different laboratories (R), may be expected to lie with a probability of 95%.

Actually we are more interested in the 5% probability that these values will be exceeded, because that is interpreted as an indication that the difference observed cannot be attributed satisfactorily to errors in the test method, and that there may be cause to search for some explanation.

But a probability of 5% is wishful thinking, because s_r and s_R are only estimates and as such may be associated with probabilities that differ from 5%.

If s is any estimate of σ with f = 10 degrees of freedom, we have by Table 5:

(53) $$\Pr(0.699 < \sigma/\underline{s} < 1.75) = 95\%, \text{ or}$$

(54) $$\Pr(1.14 \times 2^{1/2} \times \sigma < 2 \times 2^{1/2}\,\underline{s} < 2.86 \times 2^{1/2} \times \sigma) = 95\%.$$

Now

(55) $$\Pr(|x_1 - x_2| > 1.14 \times 2^{1/2} \times \sigma) = 25.4\% \text{ and}$$

(56) $$\Pr(|x_1 - x_2| > 2.86 \times 2^{1/2}\,\sigma) = 0.4\%$$

Designating by α the exceedance probability associated with r and R, these probability statements combine into a confidence interval

(57) $$\text{Conf}(0.4\% < \alpha < 25.4\%) = 95\%,$$

which holds when s_r or s_R are based on f = 10 degrees of freedom.

Under the same conditions we find with the aid of Table 5

(58) $\text{Conf}(2.4\% < \alpha < 10.7\%) = 50\%.$

These are wide intervals and bearing in mind that α is meant to be approximately 5%, (57) is highly asymmetric. For a confidence of 99% the situation will be even worse.

Of course the intervals could be narrowed by using larger numbers of observations. For r this will only need some more replicates in each participating laboratory, which can easily be organized; but for R it would require the collaboration of a larger number of laboratories and that is not such a simple matter.

Further, these intervals presuppose that we are dealing with data with normal distributions, that the σ_r's are identical for all participating laboratories, and that outliers are absent; all 3 are questionable assumptions. Hence the confidence intervals are only mentioned to illustrate the order of the uncertainties we have to take into account. They show that such quantities as r and R should be used with great caution in drawing conclusions or taking decisions.

For example, in "Standard Methods for Testing Tar and Its Products" (8), where r and R are systematically applied, it is every time stated that: "Duplicate results submitted by the same operator should be *considered suspect* if they differ by more than r.," or "Single results submitted by each of 2 laboratories should be *considered suspect* if they differ by more than R.," numerical values of r and R being given in each case. More precise assertions are certainly not justified. Whether a suspect difference has to be followed by any further steps must be decided in each case individually.

25. Some Final Remarks

In the foregoing sections we have attempted to survey the basic principles of collaborative studies as defined in section 15. There are many additional problems that have to be considered and have not been discussed. We mention a few:

The number of levels (or materials) to be included, the number of laboratories, and the number of replicates to be carried out by each of these, have to be discussed beforehand and will depend on many different considerations.

Another problem is at what stage of the development of a standard test method a collaborative study should be organized—at the conclusion of the validation when a draft standard has been established or some time after the standard has been finalized, is freely available, and in general use. ISO 5725 recommends this second policy, but it is also attractive to end up the validation with a collaborative study as a final check on the draft standard, and in order to include values of s_r, s_R or r, R in the final version.

Further, given that the levels of the test property can vary within wide limits, and that consequently quite a number of different levels have to be used, the fitting of a regression of s_r and s_R on $\bar{x}$ may need a more detailed consideration.

And in actual applications we may often have to compare average test results from different laboratories, and r and R, which only refer to single test results, may have to be modified. Some of these questions are discussed in the standard ISO 5725, and we must leave it at that.

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Outliers in Collaborative Studies: Coping with Uncertainty

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An overview is given of the options available in detecting and dealing with outliers in collaborative studies. The fundamental points of agreement and disagreement are highlighted. The common sense approach of just looking at the data is emphasized. The importance is stressed of making a harmonized choice of outlier treatments, even though such a choice may not be optimal for all circumstances.

A major purpose of an interlaboratory collaborative study is to test an analytical chemical method and thereby to estimate its variability. Such variability has 2 important aspects: *(1)* a within-laboratory variability, expressed as the repeatability standard deviation (s_o); and *(2)* an among-laboratories variability, typically expressed as the reproducibility standard deviation (s_x).

Related to these 2 standard deviations are the repeatability relative standard deviation (RSD_o) and the reproducibility relative standard deviation (RSD_x), which equal (100 times s_o or s_x) divided by the average concentration.

These variability parameters are reported in AOAC publications. The values are estimated by a conventional one-way analysis of variance treatment, using the "components of variance" derived from the mean of the sum of squares within laboratories and the mean of the sum of squares between laboratories. The optional routine VARCOMP in the well known SAS® (Statistical Analysis System) computer package can perform the computations as can the FDA in-house APL-language program, FDACHEMIST. Programs in the BASIC language have also been written to estimate s_o, s_x, RSD_o, and RSD_x from collaborative study data. When one is dealing with a balanced design (i.e., identical number of values furnished by each participating laboratory), simple hand calculations can be used by following the prescription given in the *Statistical Manual of the AOAC* (1) or in certain standard statistical textbooks (2, 3).

Analysis of variance, with its over half a century of vigorous existence, provides a kind of Esperanto for describing variability in analytical chemical methods. The mathematical steps and the statistical interpretations of these variability parameters are universally accepted. As far as the mere mechanism of computation of these parameters and the physical meaning ascribed to them are concerned, harmonization already exists among scientists and statisticians in general and among the International Organization for Standardization (ISO), AOAC, and the American Society for Testing and Materials in particular.

ISO has contributed the concept of repeatability confidence values and reproducibility confidence values, denoted r and R, respectively, and equal to 2.83 times s_o and s_x, respectively. These quantities, intuitively appealing to chemists, are maximum tolerable differences between 2 successive values from the same laboratory or from 2 different laboratories. These secondary quantities are directly related to the more conventional variability parameters and are freely interchangeable with them. Therefore, harmonization is not hindered by the introduction of these ISO values, unfamiliar as they may be to non-ISO analysts.

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When certain trivial notational discrepancies are eliminated, complete harmonization will be achieved in the estimation procedure for measures of variability for collaboratively studied analytical chemical methods.

Inconsistencies: Procedural and Statistical

Once valid data are available, complete agreement already exists on what the data will yield as estimates for the repeatability and reproducibility parameters. The problem thus lies in ascertaining just what constitutes valid data. Differences exist, both procedural (how to perform a collaborative study) and statistical (how to purge incorrect or faulty values from those provided by the collaborating laboratories).

For example, inconsistencies exist in what sort of analytical method is subjected to a collaborative study. Generally, ISO chooses already tested and approved methods, while AOAC tends to use collaborative studies as a prerequisite intermediate phase in method approval. Further differences exist, even among AOAC collaborations, in the nature of the collaborating laboratories selected. Some studies use only the "best qualified" laboratories, others use any laboratory that volunteers to do the work, and still others screen these volunteers on the basis of practice samples.

These inconsistencies can lead to ambiguity in comparing the variabilities of different chemical methods. Some of the above procedures are intrinsically more variability-prone than others, thereby hindering direct comparisons across studies. For example, the estimated RSD_x of a method investigated by the best-qualified-laboratory criterion would be expected to be better (i.e., smaller) than the estimated value for this parameter when the "any volunteer" criterion is invoked for a similar method.

Reconciling these procedural inconsistencies is of concern to those who want harmonization and compatibility among collaborative studies. Different needs and unavoidable constraints lead to these procedural inconsistencies, with the result that such inconsistencies may be inherent and therefore must be accommodated. One path (of many) toward this accommodation is to establish a taxonomy for the differing procedural approaches and thereby to indicate along with the statistical results the appropriate "name" for the criteria used to implement the details of the study. As an example, "DEVELOPMENT:PRELIMINARY/CHOICE:ANY + SCREENED" might be the name selected from a limited pre-established menu of descriptors of types of collaborative studies. At least this path offers the hope of permitting semi-quantitative comparisons by explicitly labeling estimates of variability parameters with their pedigrees.

The other major area of inconsistencies—of disharmonies—is the statistical one of identifying and rejecting outliers, where outliers may be defined as values that do not belong with the remaining bulk of the reported values from a collaborative study. Some analysts (both chemical and statistical) firmly refuse to omit any data by insisting that all values be treated on an equal footing as representative of the analytical method in actual practice. Carried to extremes, this philosophy would regard accidentally dropped test tubes as still representative of the analytical method in actual practice. A diametrically opposite school of thought calls for the rejection of any values that seem out of line. Undisciplined and wanton

application of this principle can lead to rejection of a sizeable fraction of the data and to erroneously optimistic estimation of the variability of an analytical chemical method. Most analysts strive for a happy medium between these 2 extremes.

Unlike the procedural inconsistencies, which are possibly rooted in external factors over which we have no control, these inconsistencies in outlier handling need not occur and are more amenable to human intervention. Basic to this intervention is understanding of the underlying concepts of outlier rejection, of the variability in our estimates of variability, and of the options available and their consequences.

Underlying Concepts of Outlier Rejection

With statistics, some skeptics have claimed, you can *prove* anything. Actually, quite the opposite is true: With statistics, you can *prove* nothing. Statistics deals in probabilities, not certainties.

The modus operandi of statistics as applied to outlier detection is as follows:

(1) The system under investigation is presumed innocent—i.e., the "null hypothesis" that no faulty values (no outliers) are present—is assumed.

(2) An a priori underlying distribution of readings is assumed—e.g., the values are usually assumed to follow the familiar Gaussian or bell-shaped pattern, also called the normal distribution.

(3) A specific statistic (Note: singular) is computed—based on the observations—e.g., for the highest value in a set, a Dixon statistic (= highest value minus the next highest value, all divided by the difference between the highest value and the lowest value) is computed.

(4) A probability is then calculated for obtaining this specific calculated value or worse if the null hypothesis were true and if the distribution assumption were true. The null hypothesis is rejected if this probability is too low, i.e., if it falls below some arbitrary preassigned probability value. Conventionally, the critical probability value is either 0.05 (at which level, ISO deems a candidate outlier to be a "straggler" but still to be retained) or 0.01 (an "outlier," to be rejected).

This is the basic outlier detection approach: A distribution is assumed a priori and the candidate outlier value (or values) is checked for consistency with this distribution. Such checking is achieved by means of a statistic—a quantity whose value is calculated based usually on the location of the candidate outlier. For a given a priori distribution of the values, the probability of getting this calculated value for the statistic can be determined. Should this probability turn out to be too low—so low as to make the statistical result improbable—then the candidate outlier is rejected as a true outlier.

An important point to consider, of course, is this: The alleged outlier could be truly representative of the analytical chemical method in action but the wrong distribution could have been assumed. For example, the true distribution could be a bimodal one: 2 Gaussian curves separated from each other. In fact, just such a bimodal distribution is quite evident in some of the collaborative study data presented at this second International Meeting on Harmonization of Collaborative Studies (Washington, DC, 1984). Such bimodality could occur for example *(1)* if 2 differing reference standards were employed, or *(2)* if 2 differing techniques were used. Whatever the cause, this bimodality ("2-lumpedness") would invalidate the usual outlier tests, which assume a (unimodal) Gaussian distribution.

Tests do exist, such as the Kolmogorov-Smirnov test, to check for deviation of data as a whole (in contrast to checking just individual data points) from the Gaussian distribution. However, such tests are so notoriously insensitive to true deviations that often a reasonable check is the ULTIMATE OUTLIER TEST, namely, VISUAL INSPECTION OF THE DATA. If the collaborative study results as a whole are NOT distributed in the expected Gaussian pattern, you do not have to screen the collaborative study data for outliers because ordinarily you would then have to abandon the study and investigate the possible sources of non-Gaussian behavior.

This surrender is not so drastic as it sounds. First, you should have a Gaussian distribution unless something is wrong with the analytical chemical method, with its implementation, or with the collaborative study protocol. Gaussian patterns arise when the experimentally obtained values are influenced by the simultaneous actions of many little errors, some in the positive direction, some in the negative direction. On sound theoretical grounds, the cumulative effects of these errors will be to produce a Gaussian distribution. Second, if you calculate measures of precision for a distribution that is not Gaussian, then you cannot compare these measures with those obtained from conventional collaborative studies yielding Gaussian distributions. The measures of variability from a collaborative study refer to situations with underlying Gaussian patterns (specifically, the distribution of the laboratory averages and the distribution of the readings within a laboratory). Otherwise, the calculated measures of variability cannot be interpreted in terms of percent of values expected to fall within such-and-such a range.

Thus, the outlier tests, whether used for individual values within a laboratory or for laboratory averages, are basically tests to determine if the candidate outliers are inconsistent with the assumption of a Gaussian distribution. Purging data of outliers serves to make the estimates of the measures of variability, such as s_x or RSD_x, more correct. Even with no outliers, such estimates may not be very precise.

Variability in Estimates of Variability

Like all estimates, estimates of variability have an uncertainty—a variability—associated with them. The estimation problem is especially acute when you have only a few readings (points, values) on which to base your estimates of variability. Some examples will dramatize just how variable the estimates of variability can be.

To estimate the true standard deviation of the values from a single laboratory—assuming a Gaussian distribution, of course:

(1) Fifty-two experimental values are required to ensure that the calculated standard deviation ± 25% of the calculated standard deviation will encompass the true standard deviation 99 times out of 100.

(2) If one is less demanding, being content to have the calculated standard deviation ± 50% of the calculated standard deviation encompass the true standard deviation 99 times out of 100, even then 12 experimental values would be required.

Simulation—the last resort of the theoretician—provides further examples of the uncertainty in estimates of measures of variability. When a collaborative study consisting of 6 participating laboratories, each providing 2 values, was simulated 450 times with a true RSD_x = 30% (and with a true RSD_o = 15%), one-tenth of the estimates of RSD_x fell below 20% and one-tenth of the estimates were above 40%. When the true value of RSD_x is not so large—say RSD_x = 16%, which is a reasonable value for analytical chemical methods operating in the parts-per-million region (Horwitz curve estimate)—the magnitude of the spread is still uncomfortably

large: One-tenth of the estimates in this case were below 10% and one-tenth of the estimates were above 21%.

Since the estimates of the measures of variability are so variable, an obvious question is how the uncertainty affects our outlier tests. This sword cuts both ways: The uncertainty can be used to justify ignoring outliers since "dropping outliers will not change the estimate very much in comparison with the intrinsic uncertainty of the estimate" but one can also argue for liberality in discarding outliers because "retaining true outliers will exacerbate an already bad situation." The resolution of this aspect of the outlier quandry lies in the concept of INFLUENTIAL OUTLIERS, which may be defined as candidate outlier values whose removal leads to a significant change (decrease) in the estimate of the standard deviation. Just how significant is a matter for discussion among analysts and for harmonization at future international meetings.

Options to be Considered in Outlier Detection

One category of outlier tests is the family of Grubbs tests. These may be interpreted in terms of percent change in the standard deviation when the candidate outlier is dropped. Included in this family are a test for the highest or lowest value in a set, the 2 highest or 2 lowest values in a set, the 3 highest or 3 lowest values in a set, or the highest *and* lowest values in a set simultaneously. The Grubbs test statistic for single outlying values (either the highest or the lowest value in a set) is expressed conventionally as the distance of the candidate outlier from the average of the set, this distance being then divided by the calculated standard deviation of the original set. This Grubbs statistic, which statisticians would label as a "Studentized distance," can be reexpressed in terms of percent reduction in the standard deviation of the set of values so that you could derive a table of critical percent reductions corresponding to percent reductions that would be achieved for a Gaussian distribution only 5% of the time, only 1% of the time, and so forth.

Another family of intuitively appealing outlier tests are the Dixon tests, which are "gap" tests using the values arrayed in order of their magnitude. They are based on a ratio: the distance of the candidate outlier (either the highest value or the lowest value in a set) from a neighboring value divided by some measure of the spread of the values. The neighboring value could be the nearest neighbor or the next-nearest-neighbor or the next-next-nearest neighbor. The measure of spread of all the values could be simply the range (= highest value minus lowest value) or it could be the highest value minus the second lowest value, etc. Thus, a whole spectrum of Dixon statistics can be calculated. A rational choice can be made as a function of the number of laboratory averages involved; depending on this number, tables are available indicating which of the Dixon statistics is the most "powerful," i.e., most capable of identifying a genuine outlier.

Besides the Grubbs family and the Dixon family of outlier tests, the Cochran test (not part of any family) is frequently used to flag outlying laboratories. However, the problem addressed by the Cochran test is entirely different from that addressed by the Grubbs and Dixon tests. While the Grubbs and Dixon statistics are used to identify laboratory averages that are inconsistent with a Gaussian distribution for the observed laboratory averages, the Cochran test is used to identify a laboratory whose within-laboratory variance is inconsistent with the within-laboratory variances of the other laboratories. Recall that the variance is simply the square of the standard deviation. The Cochran test statistic for a disparate variance (square of s_o) is simply the highest within-laboratory variance divided by the sum of all the within-laboratory variances. If this fraction is too large, i.e., if the probability of getting a fraction as large as the one obtained, or even larger, is too small, the laboratory with the largest s_o is deemed to be an outlier. An alternative test, the Bartlett test, is available for checking on excessive within-laboratory variance, but this test involves logarithms and is inappropriate for small values. Whether or not a laboratory is a Cochran outlier has nothing to do with whether or not the laboratory is a Grubbs or Dixon outlier; the tests check different sorts of deviations.

There should be universal agreement that these are the outlier tests to be employed, namely, Grubbs, Dixon, and Cochran. Harmonization is required, though, on *(a)* which of the subsets of these tests are to be used, *(b)* what probability values are to be considered so low as to be improbable, *(c)* how many of the values should be subjected to outlier tests, *(d)* what are the appropriate guidelines for rechecking the data once they have already been purged of outliers, *(e)* how experience with previous collaborative studies can be utilized, and *(f)* how the ULTIMATE OUTLIER TEST—that of visual inspection of the data—can be brought to bear on the problem of ensuring representative data. These questions, requiring a consistent, uniform answer, form the agenda for part of the next international meeting on the harmonization of collaborative studies.

Conclusions

General agreement already exists in the statistical treatment of the data from collaborative studies. Some notational differences persist but the only major obstacle to full statistical harmonization arises from the lack of an agreed-upon procedure for dealing with outliers.

In view of the intrinsic variability of estimates of variability, retaining or rejecting a laboratory will often make no significant difference in the final estimates. In fact, one family of outlier tests—the Grubbs tests—can be interpreted as a test that depends specifically on how much of a change is made in the standard deviation of the laboratory averages when the candidate outlier laboratories are dropped.

Choices must be made as to the types of outlier tests to be employed, the confidence levels to be applied, the conditions required for recycling (resubmitting a set of data purged of alleged outliers to outlier tests), etc. A need exists for these choices to be made soon. Statistics forms only a small portion of the apparatus of collaborative studies and outliers form only a small part of statistics. Yet, like a small pebble in a shoe, the outlier problem is irritating and demands a disproportionate amount of attention and concern. Fortunately, almost any official choice of outlier options for adoption by all will do. Admittedly, situations will arise when the formally adopted outlier options are not appropriate, but even in such cases at least a common reference point will be provided. We analysts involved in collaborative studies should not be like that medieval donkey equidistant from 2 equally tempting stacks of hay, who died of starvation because it could not make up its mind. For the sake of harmonization, it does not matter—within limits—what outlier options are finalized, but choose we must.

Other Works

Among the references most relevant to the problem of outliers in collaborative studies are the *Statistical Manual of the AOAC* (1) and ISO document 5725 (4). Only specialized books are available on the topic of outliers in general, such as those by Barnett and Lewis (5) and Hawkins (6).

The most appropriate references are a projected series of articles in *J. Assoc. Off. Anal. Chem.*, of which the first 5 have already been published (7–11). For those who want to obtain the necessary statistical background, the books by Snedecor and Cochran (12) and Sokal and Rohlf (13) are recommended. Nothing, however, is more useful in this area than practical experience and the sound judgment and good common sense that such experience imparts.

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Minimum Criteria for Validation of Analytical Methods

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Two aspects must be considered in outlining minimum criteria for validation of analytical methods: management issues and technical issues. Management needs background information and information on issues, options, and implications to identify priorities. The value of the work should be clear to ensure its support and implementation. International adoption of AOAC guidelines for collaborative interlaboratory studies would provide technical criteria for future tests.

One of the early quoted uses of the word "valid" in the English language is ascribed to the English poet and diplomat Matthew Prior, who was born in 1664 and died at the age of 57 years in 1721. After various adventures he was impeached by Sir Robert Walpole and imprisoned in London in 1716. During his 2 years in prison he wrote "Almo," or as it was better known, "The Progress of the Mind." The work contained many neat epigrams for which he was famous, and one which is particularly relevant to the title of this talk: "For when one's proofs are aptly chosen, four are as *valid* as four dozen." What better epigram could we compose except perhaps to substitute "5 × 6 are as valid as 25!"—if the proofs are aptly chosen, of course.

When I was invited to prepare this paper, Harold Egan emphasized that it should concentrate on the practical aspects which the Analytical Methods Committee (AMC) of the Royal Society of Chemistry had identified from experiences over the past several years. Much of this experience has been gained in conducting collaborative tests with methods of analysis destined for adoption into UK and EEC legislation. This aspect of AMC work accounts for about 80% of the effort and produces several completed reports per annum.

Although UK legislation has required statutory methods of analysis since the 19th century in many important areas such as agriculture and medicine to help enforce statutory declarations, this was an innovation for EEC legislation drafted in the 1970s. Even more radical was the requirement by the European Economic Community that statutory "minimum limits of variation" would be required for lengthy lists of compulsory and optional statutory label declarations. These developments have stimulated demands for soundly based official methods of analysis. The most favored means of producing the methods is collaborative tests, and there is a desire among the sponsors and organizers to harmonize minimum criteria for validation of methods of analysis. The Report of the AOAC Committee on Collaborative Interlaboratory Studies (1) published earlier this year was eagerly awaited by the AMC who intend using the proposals as the basis for their collaborative test procedures. The AMC was informed of the progress of the AOAC work and was given the opportunity to comment on the draft proposals.

I am sure that adoption of the AOAC guidelines internationally would represent a major step forward in harmonizing collaborative test procedures on a sound professional and practical basis.

I would like to comment on the practical aspects of collaborative testing as members of the AMC may see them, and two aspects are important for consideration in drawing up minimum criteria, namely *management* issues, and *technical* or statistical considerations.

Management

The basic management approach to organizing a collaborative test on a particular method would be to know (a) background information, (b) main issues, (c) options, (d) implications, and (e) proposals. With limited resources available to the AMC, this initial assessment will identify priorities and so direct the work to best effect. This approach has been used in the AMC particularly with regard to work on proposed statutory methods. The sponsors of the work, for example,

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the Ministry of Agriculture, are always involved in the management discussion which takes place.

This management approach should be regarded as one of the minimum criteria for validation—it concentrates the minds of everyone concerned on the reasons for the test and why it is necessary to address the problem in the way proposed! It helps to avoid the criticisms that could be made about different scientifically based collaborative test units as "islands of anarchy operating in a sea of chaos" (with acknowledgment to Aneurin Bevan talking about trade unions!). In particular, the orders of magnitude of acceptable variation may be specified in relation to the importance of the analytical parameters from nutritional, safety, quality, or commercial considerations. There is clearly no point in agreeing to organize a collaborative test based on a design that would be inappropriate for the known requirements. The technicalities of this aspect are discussed later.

The managers should also have a knowledge of the activities of other groups of workers, and this raises the important question of mutual recognition of collaboratively tested methods. Perhaps more effort should now be directed at improving the formal contacts on the general management of collaborative tests now that the technical guidelines seem to be nearing a successful conclusion, and, I hope, adoption by many of the organizations represented at this symposium.

Another important part of the management criteria is the role played by the professional statisticians. It is, therefore, appropriate that they review their role and ensure that where they are involved with chemists and others in managing collaborative tests that they are (a) competent in the relevant statistical theory, (b) experienced in applying that theory to practice, and (c) able to communicate both (a) and (b) to nonstatisticians. Point (c) is particularly important and reporting must not be couched in technical jargon meaningful only to other statisticians. The words "significance," "random," and "normal" are well misunderstood. The statistician must never allow the people using the statistical analysis to misunderstand the findings. Management authority is required to ensure that the results and report of a collaborative test are carried out in practice, and good communication and understanding are vital. In this respect, one should never forget that most managers with the authority to implement the results of collaborative tests will need to know the costs involved. The costs of adopting and applying the method in laboratories are usually easy to calculate. However, the costs of applying the results will depend on several factors, including the confidence limits that are applied to the results. If these are commercially significant, further work might be necessary as a planned program designed to provide more information which may allow management decisions to be made with the required confidence.

The AOAC guidelines contain much specific advice on the preliminary work before a method is studied with the emphasis on the technical factors and, therefore, the involvement of technical managers. It could be argued that support for harmonization of collaborative tests would receive added impetus and importance if general management was involved at the outset. The minimum criteria would be directed at a different category of parameters but just as important as those for the technical and statistical requirements. The general manager who is asked to support expenditure on collaborative testing might require to be reassured that (a) collaborative testing was necessary, e.g., to help formulate official methods and realistic "limits of variation"; (b) costs were acceptable; (c) implications of adoption of the method were beneficial; (d) no better alternative than participation in the test was available.

General management support can only be enlisted if the case is well thought out and presented. If such a case cannot be made, the value of the test is highly questionable and one could, therefore, argue that the "general management test" is one of the minimum criteria. Passing such a test will help to underpin the commitment of collaboration and help create the environment for a successful conclusion and utilization of the findings when the results are discussed with the "general managers."

Technical Criteria

The AOAC guidelines are the result of many years of work based on 100 years of experience. AMC's roots in collaborative testing also span 100 years! It would be a major achievement if both organizations were to pronounce that an agreed protocol was to be the basis of all future tests. It is difficult to find areas where beneficial amendments could be made which are not already identified, but practical considerations force constraints on the experimental designs, especially in the number of laboratories that should participate in a collaborative test.

It is for this reason that the minimum criteria of "(No. of materials × No. of laboratories) = 30 with a minimum of 5 laboratories providing usable data with single determinations" should be examined.

What are the "management criteria" that may be applied to this minimum technical criterion?

(a) *Background.*—The current situation in AMC's experience is that in most cases only 5–10 laboratories are able to take part in the collaborative test. The reasons are that only these limited numbers are available and willing to take part and also because the methods under test are applicable only to certain sectors of industry. If the tests were extended to cover the same industry sectors in EEC member states, then the number of potential collaborators could reach 20 to 40 for tests on animal feeds, for example.

(b) *Main issues.*—For most statutory methods for use in the European Economic Community, the most important component of the method to be measured is the interlaboratory variability or reproducibility. This is required as a basis for stating the limits of variation that may be applied to reported results and that are important in helping decide the limits of variation that are allowed for label declarations, e.g., proteins or feed additive level. It is worth noting that the AOAC minimum criteria do not permit an estimation of repeatability to be made since only single determinations are specified. The repeatability is sometimes quoted in EEC methods as a guide for the analyst!

(c) *Options.*—Assuming that a collaborative test provides the only source of information on reproducibility, then the number of laboratories taking part is of key importance. A knowledge of the reproducibility of a method of analysis is important essentially because it permits a distinction to be drawn between those differences—whether from a specified figure or between results in different laboratories—which can reasonably be ascribed to chance and those which cannot. A statistical test of significance using the estimated standard deviation is implied. The reliability of an estimated standard deviation is dependent on the number of degrees of freedom on which the estimate is based, i.e., for a reproducibility standard deviation, effectively on the number of participating laboratories where only a single sample is involved for analysis in each laboratory.

As an example of the uncertainty of estimates based on limited numbers of degrees of freedom we may note that an estimate based on 10 degrees of freedom (11 participating laboratories) may reasonably be expected to vary, i.e., have 95% probability limits, between 0.70 and 1.75 times the true standard deviation which the estimate purports to measure. Reference is made in Section 2.5.3. of the AOAC guidelines (1) to these uncertainties and the following table helps to emphasize the point.

Degrees of freedom	*95% confidence interval for true standard deviation*
1	(0.45s; 31.9s)
10	(0.70s; 1.75s)
30	(0.80s; 1.34s)
40	(0.82s; 1.28s)
100	(0.88s; 1.16s)
400	(0.94s; 1.07s)

In making a single test of significance based on an estimated standard deviation it is perfectly legitimate to allow for the uncertainty of the standard deviation estimate by carrying out a t-test. For moderate numbers of degrees of freedom the percentage points of the t-distribution begin to approach those of the normal distribution, e.g., for 9 degrees of freedom the upper 2.5% point of the t-distribution is 2.26 compared with 1.96 of the normal distribution, and for 30 degrees of freedom it is 2.04.

It is this relatively rapid approach to an approximate normality which has presumably led to the idea that only a moderate number of participating laboratories is necessary in a collaborative test. However, when repeated significance tests are carried out using the same estimated standard deviation, the conclusions are not independent and the proportion of false conclusions within the set that is generated may then depart markedly from the significance level of the test.

The risk of wrongly concluding a real difference where none exists, an error of the first kind, is very dependent on the quality of the estimated standard deviation. It is clearly important not to provoke unnecessary dispute nor unnecessarily to blunt comparisons. This implies that we must seek to define the reproducibility standard deviation closely enough for the risk of error of the first kind not to depart too markedly from the nominal figure.

Suppose we seek to ensure (with 95% confidence) a nominal "1 in 20" risk is not worse in practice than 1 in 10. We then find that our estimate must be based on at least 40 degrees of freedom and one could argue for at least 41 participating laboratories. With different prescriptions, one arrives at different numbers but if we wish to avoid publishing estimates that may rapidly fall into disrepute, they must all be based on at least several tens of degrees of freedom.

For most of the methods currently being studied by the AMC, the number of participating laboratories is usually less than 10, and one way to increase the numbers as already stated, is to seek collaboration on an international scale. This is already happening in the testing of feed additives where a number of EEC-based laboratories have joined in AMC tests. Contacts are also developing with AOAC workers.

The other obvious way to increase the degrees of freedom is the circulation of a larger number of samples in an attempt to make up by replication what is lost by lack of coverage.

The extent to which this will be successful is almost wholly dependent on how far the between-laboratories component of variation is random over time and samples, i.e., can be represented by a laboratories × samples interaction, because it is only the degrees of freedom for the latter that can be increased by replication.

It is preferable for each laboratory to analyze samples on different days because in this way the between-days component is included in the laboratories × samples interaction. If the samples were all analyzed on one day in each laboratory, the component would be included in the between-laboratories variance which is not so well estimated. (See Appendix I.) Section 1.3.1. of the AOAC guidelines alludes to this but does not specifically advise it.

Where the collaborative test has to be conducted with a minimum of 5 laboratories, for example, it is implicit that there are only minor interlaboratory biases. While the conventional definition of reproducibility draws no distinction between systematic and random between-laboratory effects, there is the presumption of a population of interlaboratory differences from which it is possible to sample at random and for which worthwhile probability statements can be made on the basis of a calculated standard deviation. Thus, where only a small number of laboratories are concerned, and the differences between them are persistent, it may be more useful to view collaborative work in the first instance as a means for the identification and progressive elimination of interlaboratory biases, i.e., long term systematic differences. If interlaboratory biases do not contribute substantially to the overall uncertainty, it is possible to establish a meaningful reproducibility standard deviation primarily on the basis of a laboratories × sample interaction, so that the small number of participants is no longer a limitation.

However, any collaborative test involving only a small number of laboratories cannot be regarded as a quick "once off" operation, and needs close statistical monitoring throughout, as discussed below.

(d) *Implications.*—The proposal that the minimum criterion (of 5 laboratories × 6 samples) can form the basis of collaborative testing has a number of important implications. The validity of the data should be the responsibility of a professional statistician conversant with the design and experienced in its interpretation. This is particularly necessary when tests are being conducted at the minimum limits regarded as acceptable with the important qualifications already discussed. The validation of a method implies that it will either be accepted without further study—or rejected. The primary consideration is reproducibility, so it is of interest to consider the 5 laboratories × 6 samples proposal from an acceptance sampling viewpoint.

A reasonable and objective rule would be to accept a method if the estimated reproducibility was less than a stated critical value. Otherwise the method would be rejected or recommended for further studies.

Considering the analysis of variance table we have the following:

Source of variance	*Degrees of freedom*	*Observed mean square*	*Expected mean square*
Laboratories	4	MS_L	$\sigma_0^2 + \sigma_{LS}^2 + 6\sigma_L^2 = EMS_L$
Labs × samples	20	MS_{LS}	$\sigma_0^2 + \sigma_{LS}^2 = EMS_{LS}$

(Using Steiner's notation)

The reproducibility variance σ_x^2, which is $(\sigma_L^2 + \sigma_{LS}^2 + \sigma_0^2)$, is estimated by

$$s_x^2 = (5 \times MS_{LS} + MS_L)/6$$

The distribution of this statistic depends on the true reproducibility variance, σ_x^2, and also on the size of σ_L^2 relative to $\sigma_0^2 + \sigma_{LS}^2$. Greater precision of estimation is obtained when σ_L^2 is relatively small because this reduces the contribution of MS_L which is based on only 4 degrees of freedom.

If one accepts that analyses should be done on different days for the reasons shown in Appendix I, then let:

$$p = \sigma_L^2/(\sigma_0^2 - \sigma_{LS}^2 + \sigma_D^2 + \sigma_L^2) = \sigma_L^2/\sigma_x^2$$

This represents the square of the correlation between analyses of different samples by the same laboratory on different days.

If p is specified then the distribution is known since:

$$s_x^2 \sim \sigma_x^2 \left\{ \frac{5(1-p)}{6} \cdot \frac{\chi^2_{20}}{20} + \frac{(1+5p)}{6} \cdot \frac{\chi^2_4}{4} \right\}$$

where χ_ν^2 denotes a chi-squared variate with ν degrees of freedom.

From data such as that described by Horwitz et al. in 1980 (2) it should be possible to specify the level of reproducibility that might be achieved by adequate methods. A value could be specified, say σ^2_{worst} such that there should be a high probability of rejection if σ_x^2 exceeds this value.

The extreme situations are:

$$p = 0 \text{ when } s_x^2 \sim \sigma_x^2 \cdot \frac{\chi^2_{24}}{24}$$

which is the best situation where there is no interlaboratory variation, i.e., the same variance as for 25 samples in one laboratory, and

$$p = 1 \text{ when } s_x^2 \sim \sigma_x^2 \cdot \frac{\chi^2_4}{4}$$

which is equivalent to 5 observations with laboratory biases the same across all laboratories.

The graphs in Appendix II give the probability of acceptance plotted against true reproducibility/critical estimate. Explanatory notes illustrate how the graphs may be used. These show that for reasonable performance, methods should be accepted only if:

$$s_x^2 < 0.27\sigma^2_{worst} \quad (p = 1) \quad \rightarrow \quad 0.65\sigma^2_{worst} \quad (p = 0)$$

or correspondingly:

Estimated reproducibility:

$$<0.52 \text{ worst reprod. } (p = 1) \quad \rightarrow \quad 0.81 \text{ worst reprod. } (p = 0)$$

As a consequence of ensuring a low chance of acceptance for poor methods there is a moderate chance that good methods will be rejected.

Corresponding to the above, the chance of acceptance rises to 95% for:

True reproducibility:

$$<0.33 \text{ worst reprod. } (p = 1) \quad \rightarrow \quad 0.65 \text{ worst reprod. } (p = 0)$$

Clearly the performance of such a procedure in practice depends on the distribution of actual reproducibility variances of methods tested relative to σ^2_{worst}.

Consideration of this distribution would provide an objective basis for judging the adequacy of the 5 laboratories × 6 sample design.

Conclusion

The minimum criteria for validation of methods of analysis by collaborative testing have 2 basic aspects: management and technical.

The management criteria should be based on considerations that would normally be applied to any important investment where people, time, and money are involved! The value of the work should be clearly identified to ensure that it is adequately supported and implemented.

The technical criteria have been published by the AOAC (1) and deserve widespread international support. The minimum criteria (5 laboratories × 6 samples) is acceptable provided the design is objectively assessed and shown to be adequate. This can be done and may involve "quality of method" control tests and provision for updating the reproducibility estimates.

The means are now firmly established whereby collaborative test managers can make soundly based decisions for the acceptance of methods and avoid poor methods coming into use by adopting efficient designs backed by good management.

Acknowledgments

I would like to acknowledge the contribution made by statisticians who advise the AMC, and in particular L. J. Aspinall of Unilever Research Laboratories and W. Spendley of I. C. I. Agriculture Division, who have assisted in the preparation of this paper.

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APPENDIX I

5 Laboratories × 6 Samples

Analysis of samples all on 1 day compared with analysis on different days.

	Expected Mean Square	
	(1) Analysis on same day	(2) Analysis on different days
Labs	$\sigma_0^2 + \sigma_{LS}^2 + 6(\sigma_L^2 + \sigma_D^2)$	$\sigma_0^2 + \sigma_{LS}^2 + \sigma_D^2 + 6(\sigma_L^2)$
Labs × samples	$\sigma_0^2 + \sigma_{LS}^2$	$\sigma_0^2 + \sigma_{LS}^2 + \sigma_D^2$

The same combinations of mean squares provide s_x^2 but (2) provides greater precision since the contribution of MS_L is less. From a commonsense viewpoint, option (2) is preferable in that it gives a broader basis for inference.

APPENDIX II
Acceptance Criteria

The graphs (Figure 1) show the probability of acceptance plotted against the ratio true reproducibility/critical esti-

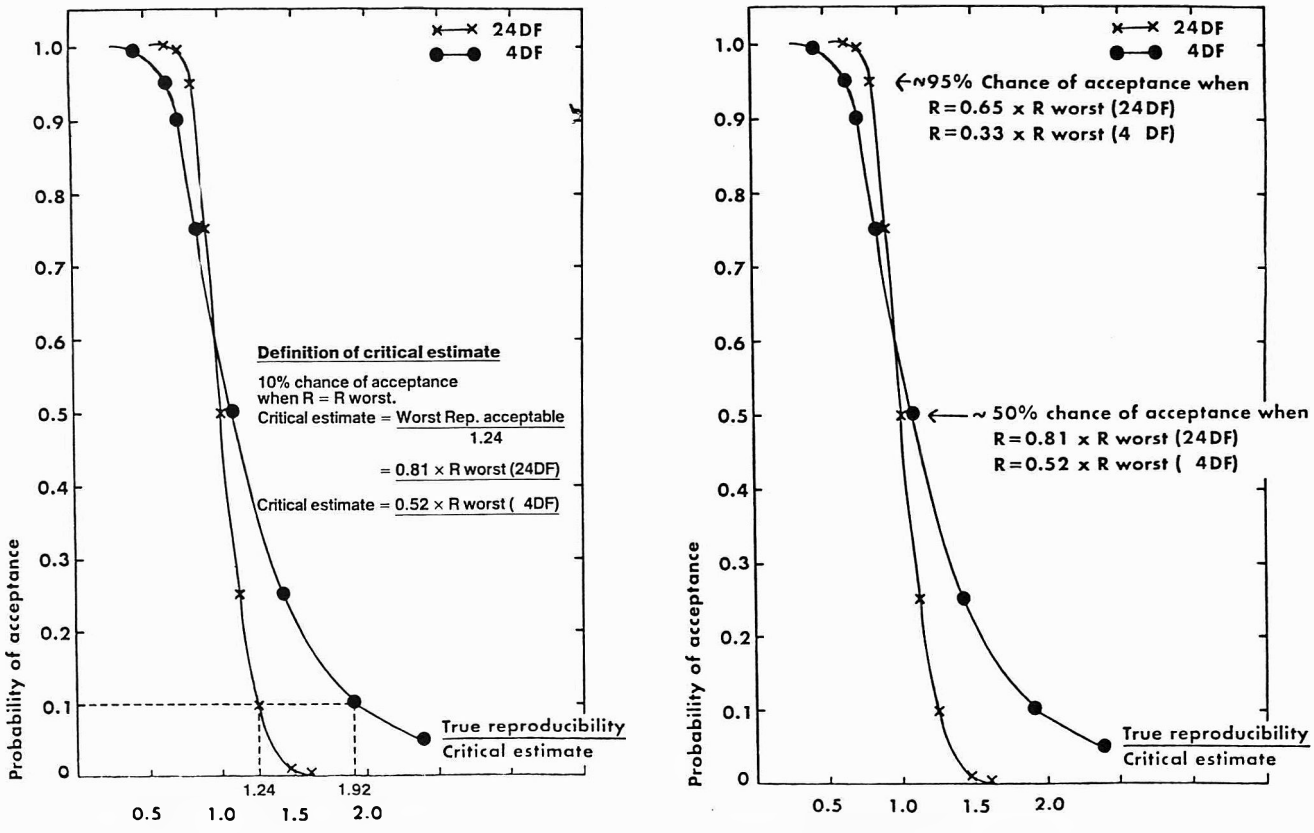

Figure 1. Graphs of probability of acceptance vs true reproducibility/critical estimate.

mate—remembering that we have decided to accept a method if the estimated reproducibility was less than a stated critical value.

It is worth noting with respect to the graph that because it has been expressed in terms of a ratio of reproducibility it applies to

(1) the AOAC definition σ_X
(2) the ISO definition $2\sqrt{2}\,\sigma_x$
(3) reproducibility coefficients of variation since these differ by scale factors

We first have to define the critical estimate, and we could set a standard that we will have a 10% chance of acceptance when the true reproducibility is equal to the worst reproducibility we will accept.

$$R = R_{worst}$$

From the graph, the critical estimate of a 10% chance of acceptance is calculated as

$$R_{worst}/\text{critical estimate} = 1.24$$

Therefore, critical estimate = $0.81 \times R_{worst}$ for 24 degrees of freedom and critical estimate = $0.52 \times R_{worst}$ for 4 degrees of freedom.

If we now want a 95% chance of acceptance of a method which will satisfy our criterion that the estimated reproducibility will be less than a stated level, then

$$\begin{aligned} R &= 0.81 \times \text{critical estimate} \\ &= 0.81 \times 0.81 \times R_{worst} \\ &= 0.65\ R_{worst} \text{ for 24 degrees of freedom} \\ &= 0.33\ R_{worst} \text{ for 4 degrees of freedom} \end{aligned}$$

For example, say that we will accept a method if the reproducibility is no greater than 10 (R_{worst} = 10). Then we have a 10% chance of acceptance if the estimated reproducibility measured in our test (true reproducibility) = 10.

If the estimated reproducibility is 8.1, then we have a 50% chance of acceptance of the method.

If the estimated reproducibility is 6.5, then we have a 95% chance of acceptance, assuming there are only minor interlaboratory biases and that the estimate is based on 24 degrees of freedom.

In accepting this approach, 2 aspects of reproducibility estimates have been considered: (a) their precision related to their use in practice, and (b) their use for screening poor methods. Adequate performance for (b) is a minimal requirement for a validation exercise.

Given the practical constraints, more information may be needed for the first aspect (a) than is appropriate for validation. This is where the idea of updating estimates as a secondary function of "quality of method" monitoring fits in.

CHEMICAL CONTAMINANTS MONITORING

Enzyme Immunoassay-Based Survey of Prevalence of Gentamicin in Serum of Marketed Swine

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Sera from 3182 swine from a national sampling were tested in the gentamicin enzyme immunoassay. Of the sera tested, 6 (0.19%) contained gentamicin. Only 1 serum may have been associated with muscle levels above the tolerance. During the survey, a single analyst processed 300 samples daily. The immunoassay survey was an effective and economical method of obtaining information on the prevalence of a residue.

A large number of drugs, pesticides, or adulterants could conceivably find their way into the food supply. For residue control programs, the analysis of every sample for all possible residues is not a realistic goal. The sheer number of chemicals precludes routine analysis for every possible contaminant. Programs designed to monitor the meat supply for the presence of environmental contaminants and drug residues must establish priorities for analysis. The selection of priorities requires a reliable data base for assessing the potential hazards and estimating the probability of finding a given residue in the tissues of marketed animals. The results obtained through surveys of the prevalence of residues in marketed animals could be a valuable source of information useful for establishing regulatory priorities.

In 1983, gentamicin was newly approved for use in swine. The drug was approved as an injectable in 3-day-old piglets for neonatal diarrhea (21 CFR 522.1044) and as an oral solution in drinking water for neonatal diarrhea or for swine dysentery (21 CFR 520.1044a). The new approvals were likely to be accompanied by an increasing frequency of gentamicin use. The months following these new approvals seemed like an excellent time for a survey.

Sera from the Animal and Plant Health Inspection Service (APHIS)/Food Safety and Inspection Service (FSIS) Pseudorabies/Trichinellosis Slaughter Survey were available. These sera were collected according to a statistically designed pattern to reflect the sera of swine brought to slaughter in the United States at the time of sampling. The availability of the sera made the gentamicin survey achievable without the cost and time required to arrange and implement a special sampling.

The enzyme immunoassay used for the survey is an adaptation of the assay developed by Standefer and Saunders (1). As used in this survey, the method can detect 2.3 ng/mL (2.3 ppb) of gentamicin in undiluted swine serum. The procedure is simple and rapid. Two-hundred to 400 samples were processed daily by a single analyst.

Experimental

Sampling Method

The serum samples used in this survey were obtained from a national sampling of swine serum collected by APHIS and FSIS to determine the prevalence of pseudorabies and trichinellosis in swine. The samples were selected on the basis of a stratified random sample design and should be representative of the swine coming to market in the United States at the time of collection (the gentamicin samples were collected between February 28 and October 1, 1983). The gentamicin survey used samples in the same sequence in which they were numbered by APHIS. Details of the sampling can be obtained from V. C. Beal, Jr, Veterinary Services, APHIS, Hyattsville, MD 20782.

Reagents and Equipment

Rabbit antigentamicin antibody (Lot A150) was purchased from Western Chemical Research Corp. (2300 N Highway 287, PO Box 1255, Ft Collins, CO 80522). Western Chemical reported that the antibody showed 33% cross-reactivity with sisomicin and 4% with netilmicin. The aminoglycosides commonly used in agriculture did not cross-react. The coating buffer was 0.01M carbonate buffer, pH 9.6, containing 0.02% sodium azide. The buffer was prepared by mixing 16 mL 0.2M sodium carbonate, 34 mL 0.2M sodium bicarbonate, and 10 mL 2% sodium azide. The volume was adjusted to 1 L with distilled water. The cuvet wells (Cuvette Paks®, Gilford Instrument Laboratories, Inc., Oberlin, OH 44074) were coated with 0.25 mL of a 1/12,500 dilution of the antigentamicin antibody in coating buffer. The antibody-loaded wells were stored at room temperature in a water vapor-saturated chromatography tank. Wells could be stored this way for at least 1 month. They were stored not <24 h before use. The washing solution was 0.05% Tween 80 (polyoxyethylene sorbitan monooleate, Sigma Chemical Co.) in water.

The conjugate was made by linking gentamicin to horseradish peroxidase according to method II of Nakane (2). In this method, the aldehydes produced by the periodate oxidation of the horseradish peroxidase carbohydrate moiety form a Schiff base with the gentamicin amino groups. The conjugate was prepared by Richard Brake of Los Alamos National Laboratory, Los Alamos, NM. Fresh 1/4000 dilutions of the conjugate were prepared daily for use in the assay.

The peroxidase substrate, ABTS (2,2′-azino-di-[3-ethylbenzthiazoline sulfonate (6)]), was obtained from Boehringer Mannheim Biochemicals (7941 Castleway Dr, PO Box 50816, Indianapolis, IN 46250). A 40mM stock solution was prepared by dissolving 0.549 g diammonium salt of ABTS in distilled water. A 0.55M solution of hydrogen peroxide was prepared by diluting 0.5 mL 30% H_2O_2 with 7.5 mL water. The latter 2 solutions were stored at 4°C in amber bottles. The 30% peroxide solutions available from J. T. Baker Chemical Co. or Fisher Scientific Co. were satisfactory. The 0.05M citrate buffer, pH 4.0, was prepared by mixing 330 mL 0.1M citric acid with 170 mL 0.1M trisodium citrate. The mixture was diluted to 1 L with water.

A working substrate solution was prepared daily and was maintained in an amber bottle at room temperature during the course of the day. The working substrate solution was made from 1 mL 40mM ABTS, 0.3 mL 0.55M H_2O_2, 0.1 mL 20% Tween 80, and 98.6 mL 0.05M citrate buffer, pH 4.0.

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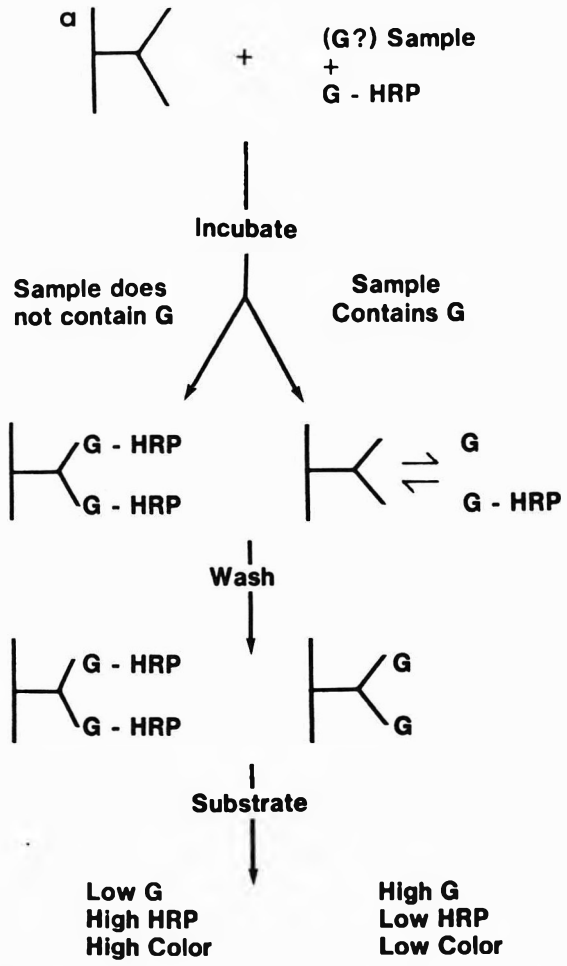

Figure 1. Gentamicin enzyme immunoassay scheme. Drawing at "a" represents antigentamicin antibody bound to plastic wall of well.

Gentamicin was obtained from Sigma Chemical Co. Standard solutions were prepared using the potency estimate provided by Sigma. Gentamicin adsorbs to glass and, to a lesser extent, to plastic. Care should be taken to be sure dilute solutions are not exposed to large surface areas (3). A concentrated solution of gentamicin (1 mg/mL) in distilled water was used to make the desired gentamicin standard solutions in serum. The standard solutions were made at concentrations of 5000, 50, and 5.0 ng/mL serum. The same serum with no gentamicin was used as a blank. Standard curves were prepared by carrying the standard solution through the assay simultaneously with the samples.

Assay Method

The gentamicin enzyme immunoassay is a modification of the assay of Standefer and Saunders who used 96-well microtiter trays (1). We adapted the assay to the Gilford EIA PR-50 Processor/Reader. The assay is shown diagrammatically in Figure 1. Antigentamicin antibody is bound to the wall of the Gilford cuvet. Gentamicin in the serum sample competes with the gentamicin-horseradish peroxidase conjugate for occupancy of the wall-bound anti-gentamicin antibody binding sites. Unbound conjugate is washed out, and the conjugate remaining is determined by measuring the enzyme activity. The enzyme activity measured is proportional to the amount of gentamicin peroxidase conjugate which remains bound to the vessel wall. The amount of conjugate remaining is inversely proportional to the amount of gentamicin in the sample:

$$\text{enzyme activity} \propto \text{wall-bound gentamicin peroxidase conjugate} \propto \frac{1}{\text{gentamicin in serum sample}}$$

To perform the assay, the antibody-coated wells were subjected to a 5-cycle wash on the Gilford EIA PR-50 apparatus and "smacked dry" on a paper towel. Each well was loaded with 0.15 mL blank, serum, or standard. Then 0.15 mL of a 1/4000 dilution of the gentamicin-horseradish peroxidase conjugate was added. The wells were shaken to mix the contents and incubated at room temperature for 10 min. The wells were washed and dried as described above. Substrate, 0.3 mL, was added to each well, and after a 7.5 min incubation at room temperature on a reciprocal shaker, the absorbance was read by the Gilford EIA PR-50 apparatus with a 405 nm filter.

Survey samples were analyzed in trains of 100 Gilford wells. The trains of samples were interspersed with blanks and standards so that each train of 100 cuvets contained 9 blanks, 12 standards, and 79 serum samples. Samples which had absorbance values distinctly less than the absorbance of the surrounding samples (corresponding to higher gentamicin levels) were tested again in another run. An example of a survey run is given in Figure 2. The scatter of samples is wide, but only one, the sample in position 8-3, was distinctly different from the general sample population. This sample was tested again in another experiment. The absorbance was low again, and the gentamicin concentration was estimated at 2.9 ppb. The sample was distinguished, not only because it had a low absorbance, but also because its absorbance was different from the absorbance of the neighboring samples.

The samples in positions 6-2, 6-3, and 6-4, in Figure 2, are distinguished by absorbances well above the population average (implying gentamicin levels less than zero). High absorbance values were associated with extensively hemolyzed samples. We found that in these samples peroxidase activity from the blood caused increased color formation. This could be eliminated by treating extensively hemolyzed sera with sodium azide before the assay. The high values probably resulted from peroxidase released from the white cells.

Calculations

Gentamicin concentrations were determined using the logit transformation (4). Logit-log paper was used to plot B/B_o against the log of the gentamicin concentration. A standard curve for gentamicin is shown in a logit-log plot in Figure 3. The concentration of gentamicin for each sample was obtained by projecting from $B/B_o \times 100$ on the ordinate (logit scale) to the standard curve for the run, and then to the concentration (ppb) on the abscissa (log scale).

Results

The standard curve in Figure 3 was constructed using swine serum fortified with known levels of gentamicin. The correlation for the logit-log line obtained by linear regression was 0.997. The gentamicin levels of the fortified sera calculated from the standard curve, and the 95% confidence limits for gentamicin levels of 10 ng/mL and lower are given in Table 1. Defining the limit of detection (LOD) for an assay as the level at which the signal is different from the blank signal at the 99.9% level of confidence (5), the LOD for the gentamicin standard curve in Figure 3 is 0.84 ng/mL. The fortified sera were prepared from the blank serum obtained from a single animal. The LOD for the survey was higher because the animal-to-animal standard deviation of the blank is larger than

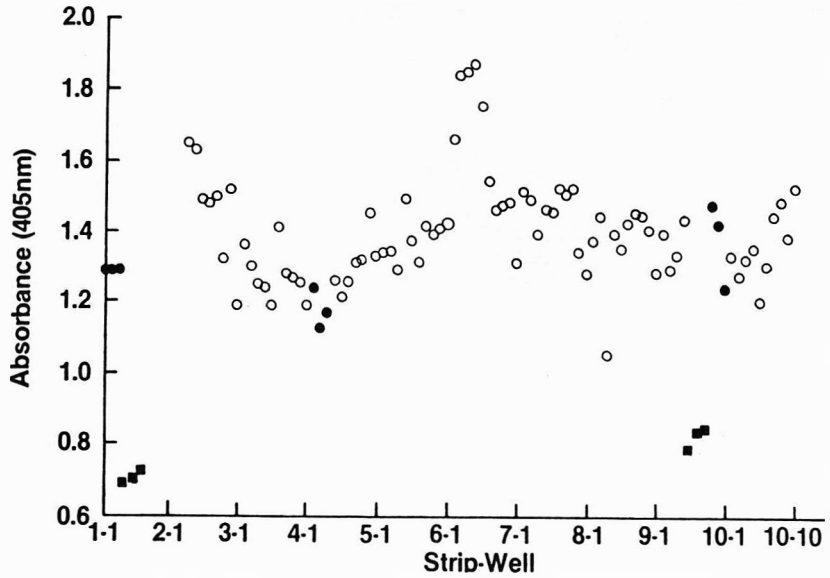

Figure 2. Sample run. Absorbance is plotted against strip-well number. Solid circles are blanks. Solid squares are 5 ppb standards. Absorbance of 50 ppb standards was too low to be shown in this figure.

the standard deviation obtained using fortified serum from a single animal. For the 79 animals tested in the experiment shown in Figure 2, the LOD was 1.8 ng/mL. In a similar experiment with another set of samples, the LOD was 2.7 ng/mL. The average of these 2 values set the LOD for the survey at 2.3 ng/mL.

The experiment-to-experiment CV was 8.8%. In 13 experiments performed on 9 different days, the determined value of a 10 ng/mL standard was 9.5 ng/mL and the coefficient of variation was 8.8%.

A serum depletion experiment was conducted to demonstrate that gentamicin could be detected in the blood of animals arriving at an abattoir 2 to 4 days after the administration of the last dose of gentamicin. This would be the most likely time of slaughter if a sick animal were treated, failed to show improvement, and was marketed. Three sows were dosed intramuscularly twice daily with 2 mg gentamicin/lb: one animal was dosed for 3 days, one for 2 days, and one for 1 day before sampling. Serum samples were collected from the 3 sows twice daily, beginning 18 h after the administration of the last dose. The serum gentamicin levels were determined by the enzyme immunoassay and the results are shown graphically in Figure 4. The serum depletion experiment indicated that gentamicin could be detected in serum for up to 1 week after the administration of the drug, even in the animal treated for only 1 day. Drug levels were higher in the animals treated for 2 or 3 days rather than 1 day. The sow treated for 1 day had a serum level of approximately 1 ng/mL 2 weeks after withdrawal from the drug. The gentamicin levels were at 5 ng/mL or higher for 4 days after the administration of the last dose. Thus, it seemed likely that the enzyme immunoassay would detect gentamicin in the serum of swine coming to slaughter within 4 days after the administration of the last dose of gentamicin.

In the survey, sera from 3182 swine were examined for gentamicin. The highest serum level of gentamicin found in the survey was 130 ppb (130 ng/mL). Table 2 lists the 6 positive sera in order of decreasing gentamicin concentration. The concentration of gentamicin in the serum which had 130 ppb was 18 times higher than the concentration in the next highest serum. The next 3 highest sera had levels of 7.1, 6.7, and 6.7 ppb, respectively.

Based on the limit of detection, 2.3 ng/mL, these 6 samples were considered positive for gentamicin. The frequency of gentamicin in the serum tested was 6/3182, or 0.19%.

Discussion

Table 2 presents the gentamicin concentrations of the 6 positive sera. The highest gentamicin level found was 130 ppb. This is about 30 times lower than the therapeutic serum level (6) or 60 to 90 times lower than the toxic serum level (7).

The other 5 positive sera had levels higher than 2.3 ppb in repeated determinations and were classified as low-positives. The cluster of 5 sera is in an interesting concentration range. This is the concentration range expected if swine are slaughtered 2 to 4 days after the last dose of gentamicin. An assay which is able to detect gentamicin in this range is required for this kind of survey.

The distribution of the positive sera within the sample subpopulations has not been examined quantitatively. Six positives are too few to allow definitive statements about the frequency of positive sera in sample subpopulations. Some information on the origins of the 6 positive sera is given in Table 2. Each of the 6 positive sera was collected on a dif-

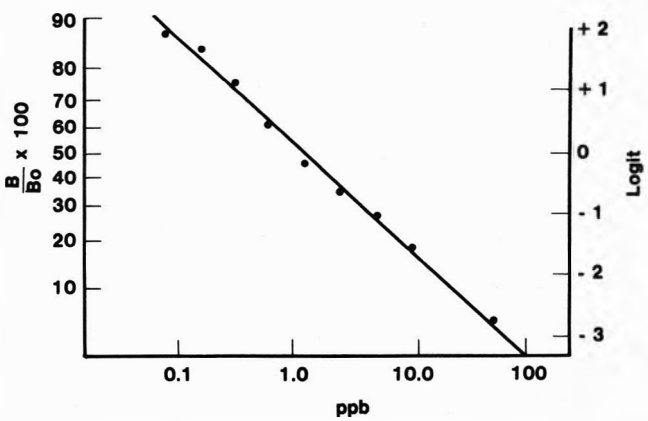

Figure 3. Gentamicin standard curve. Logit-log plot. $B/B_0 \times 100$ is plotted on ordinate on logit scale against gentamicin concentration in ppb on logarithmic scale on abscissa.

Table 1. Confidence limits for fortified serum[a]

Level of fortification[b]	Level detd	95% Confidence limits
10.0	9.41	7.96–11.4
5.00	5.00	4.18–6.06
2.50	2.80	2.48–3.20
1.25	1.57	1.36–1.79
0.625	0.65	0.56–0.75
0.313	0.26	0.24–0.28
0.156	0.12	0.092–0.150
0.078	0.093	0–0.45

[a]Expressed in ng/mL.
[b]The 10 and 5 ng/mL levels were triplicates; all others were quadruplicates.

Table 2. Gentamicin concentrations of positive samples

Gentamicin ng/ml[a] concn,	Date collected	State of origin
130.0	3/24/83	MI
7.1	4/15/83	IA
6.7	4/12/83	MN
6.7	3/07/83	IN
3.3	4/12/83	MO
2.9	5/31/83	IA

[a]ng/mL = ppb.

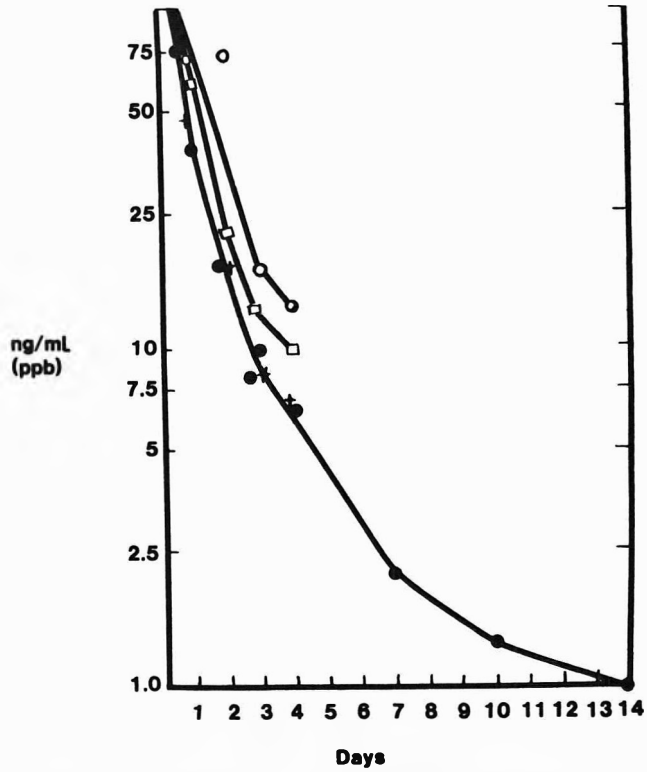

Figure 4. Gentamicin serum depletion experiment. Gentamicin levels are plotted on logarithmic scale against number of days since last dose of drug. Dot and plus sign: 1 day dosing period. Square: 2 day dosing period. Circle: 3 day dosing period. Note that origin is 1 ppb, not 0.

ferent date and each was from an animal from a different processing plant. Two animals were from Iowa. More than one sample might have been expected from some states because high-producing states and plants are represented in the sample population in proportion to their production. On the other hand, higher levels of gentamicin might be found in the serum of animals processed at abattoirs which hold animals for shorter time periods. As an example, Figure 4 shows that the animal dosed for 1 day had a serum concentration of 5.9 ppb 4 days after the last dose. Had the animal been slaughtered 2 days after the last dose, the gentamicin concentration would have been 15 ppb. In any case, not enough positive sera were detected to identify "hot spots" with any degree of certainty.

We suspected that we might find extra-label use of gentamicin in the breeding population where it might be used for the treatment of the mastitis-metritis-agalactia complex. All of the breeder sera available at the time of the survey were used; a total of 313 breeders were tested. None were positive. Our suspicion of extra-label use in sows was not supported.

The relationship between the serum gentamicin levels found in the survey and the tissue levels is complicated. Gentamicin pharmacokinetics fit a 2-compartment open model (8, 9). The model depicts a central compartment from which the drug passes into the tissue compartment or from which the drug is excreted. Drug in the tissue compartment must be returned to the central compartment for elimination. The tissue concentration is thus a function of the rate of equilibration of the tissue with the central compartment, and the central compartment concentration.

We know of no correlation of serum and tissue gentamicin levels in adult swine, but some data are available for pigeons (10) and calves (11). The pigeon and calf muscle gentamicin concentrations do not appreciably exceed the serum concentrations, but the kidney concentrations are at least 100 times higher than the serum levels. The paper by Ziv et al. (11), gives additional data for the free drug concentrations in calves at several time intervals. The calf serum and muscle drug levels were about equal, and decreased with a 4 h half-life. The kidney levels decreased more slowly, with a half-life of 11 h. Thus, as the time after the last dose increased, the ratio of kidney level to serum level increased; the kidney-to-serum ratio doubled from 516 at 4 h to 1078 at 12 h. The tenacity of the drug for kidney has been demonstrated in rats and humans, and is assumed to be related to the nephrotoxicity of the drug. These data support the expectation that kidney gentamicin levels are likely to be much higher than serum levels, but muscle levels are not expected to appreciably exceed serum levels. It is therefore possible that any of our positive samples were associated with a kidney level above tolerance, but only one serum (130 ppb) may have had a gentamicin concentration high enough to have been associated with a muscle level greater than the muscle tolerance at 100 ppb.

This study would have been strengthened by independent confirmation of the presence of gentamicin in some of the positive samples. Only the 130 ppb sample had a high enough concentration to test in the bacterial inhibition assay. Unfortunately, not enough sample was available.

A survey such as this can be an effective and economical source of data for the design of additional studies or for residue program planning. The results obtained with blanks and fortified samples, the serum depletion data, and the data from the survey itself, all contribute to a consistent demonstration of the method as a dependable information-gathering assay. Estimates of the frequency of the presence of a drug provide information on the extent of use. Coupled with serum-tissue correlations, the frequency of above-tolerance tissue level can be estimated. The simplicity and speed of enzyme immunoassays makes this technology attractive for survey use.

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Fluoroacetate Residues in Ground Squirrel and Coyote Tissues Due to Primary or Secondary 1080 Poisoning

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Fluoroacetate residues in various tissues of 1080-poisoned ground squirrels and coyotes are listed. The tissues (excluding the stomach) of squirrels poisoned with an average of 0.8 mg 1080/kg (low dose) contained from 182 to 1309 ppb fluoroacetate. In squirrels poisoned with an average of 4.8 mg 1080/kg (high dose), the tissue residues ranged from 535 to 9754 ppb fluoroacetate. Tissues from coyotes which died after consuming 1080-poisoned ground squirrels were also analyzed for fluoroacetate residues. Residues in these coyote kidneys and livers ranged from less than 10 ppb to 95 ppb fluoroacetate. The residue findings in this research indicate that a diagnostic assay for 1080 in tissues must be reliable at 10 ppb (or less) fluoroacetate.

Sodium monofluoroacetate (Compound 1080) is recognized as a potent and effective rodenticide (1). In certain cases, secondary poisoning of coyotes *(Canis latrans)* and dogs may occur due to consumption of 1080-poisoned rodents (2). Residues of fluoroacetate (FAC) from primary poisoning in coyotes orally dosed with 0.13–30.0 mg 1080/kg have been reported (3), and tissue FAC residues in field cases of 1080 toxicosis (4) were determined by a capillary gas chromatography–mass spectrometry (CGC/MS) method (5). In recent research, the risk of secondary poisoning to coyotes *(Canis latrans)*, via consumption of 1080-poisoned ground squirrels *(Spermophylus beecheyl)*, was investigated (6). The CGC/MS method (5) was used to define the FAC residues in tissues from the 1080-poisoned squirrels and coyotes. This report documents the FAC residues found in the various squirrel and coyote tissues.

Experimental

Toxicity Trials

Primary 1080 poisoning of California ground squirrels (average weight ca 625 g) was accomplished by feeding 1 g (low dose) or 6 g (high dose) of 0.05% 1080 bait (oat groats). Control squirrels received no 1080 bait and were euthanized (pentobarbital). The high dose squirrels received 3.0 mg 1080 each, the low dose squirrels received 0.5 mg 1080 each, and the control squirrels received no 1080. Various organs and tissue pools were obtained from 2 squirrels in each of the 0, 1, and 6 g 1080 bait groups and kept frozen until analyzed. The majority of squirrel carcasses were retained for secondary hazard trials with coyotes.

In the secondary 1080 hazard trials, each coyote received a squirrel at 5:00 p.m. and was left alone until 5:00 a.m. the following day. Seventeen tests were conducted where coyotes were fed 1080-poisoned squirrel carcasses. Six of the 17 coyotes died. Tissues from 3 of the 6 coyotes that died, 2 control coyotes, and one treated coyote which was euthanized were analyzed for fluoroacetate residues.

Coyote pups 1 and 2 (ca 2 kg each) consumed one control squirrel each and were euthanized (pentobarbital) 12 h after feeding. Coyote 3 (10.7 kg) consumed one low dose squirrel per day for 5 days, appeared depressed on the fifth day, and was euthanized (pentobarbital) 12 h after the last feeding. Coyote 4 (11.8 kg) consumed one low dose squirrel per day for 5 days and was found dead 12 h after the last feeding. Coyote 5 (16.2 kg) consumed 2 high dose squirrels and was found dead 12 h after feeding. Coyote 6 (17.2 kg) consumed one high dose squirrel and was found dead 12 h after feeding. Various tissues and organs were removed from each of the coyote carcasses and kept frozen until analyzed. All 1080 toxicity trials (primary and secondary) were conducted at the University of California at Davis.

Table 1. Fluoroacetate residues[a] in ground squirrel tissues[b] due to primary 1080 poisoning

Tissue	1080 Treatments: Control, 0 mg/kg	Low dose, 0.8 mg/kg	High dose, 4.8 mg/kg	High/low ratio
Brain	—	291	535	1.8
Caecum	ND[c]	1039	1567	1.5
Kidney	ND	278	1135	4.1
Liver	ND	699	1831	2.6
Stomach[d]	—	11765	55864	4.7
Spleen	ND	1309	9754	7.4
Muscle	ND	406	765	1.9
Lung	ND	182	1104	6.1

[a]ppb fluoroacetate, wet wt basis.
[b]California ground squirrels (~625 g).
[c]None detected (<10 ppb).
[d]Stomach tissue and contents.

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Table 2. Fluoroacetate residues[a] in coyote tissues due to secondary 1080 poisoning

	Controls		Low dose		High dose	
Tissue	Euth[b]	Euth[b]	Euth[b]	Died[c]	Died[c]	Died[c]
Coyote No.	1	2	3	4	5	6
mg 1080[d]	0	0	2.5	2.5	6	3
Coyote wt (kg)	~2	~2	10.7	11.8	16.2	17.2
Brain	—	—	ND[e]	ND	76	—
Kidney	ND	ND	ND	ND	95	30[f]
Liver	ND	ND	ND	ND	27	25[f]
Stomach[g]	—	ND	ND	ND	—	46[f]
Lg. int.[h]	ND	ND	—	—	140[f]	53[f]

[a]ppb fluoroacetate on wet wt basis.
[b]Euthanized with pentobarbital 12 h after receiving squirrel carcasses.
[c]Died within 12 h after receiving squirrel carcasses.
[d]Total 1080 dose(s) to squirrel(s) consumed by coyote.
[e]None detected (<10 ppb).
[f]Average of duplicate analysis.
[g]Without contents.
[h]Large intestine and ingesta.

Residue Analysis

All tissue samples were homogenized with dry ice prior to analysis. FAC analysis was done by a CGC/MS method (5) with tungstic acid extraction of the tissue homogenate, followed by partitioning of FAC into ethyl acetate. The ethyl acetate was evaporated and the residue was reacted with pentafluorobenzyl bromide (PFBBr) to produce a derivative (PFB-FAC) for CGC/MS analysis. The FAC recovery for each sample was quantified by use of ^{14}C-FAC spikes (10 ng/g tissue). The detection limit is estimated to be 10 ng FAC/g tissue (10 ppb). Other quality control factors included liquid scintillation count-verified ^{14}C-PFB-FAC derivative standards plus internal standard (dibromobenzene) monitoring of CGC/MS performance. All FAC residue analyses were done at North Dakota State University.

Results and Discussion

Fluoroacetate Residues in Squirrel Tissues

The FAC residues in the primary 1080 poisoned squirrels are given in Table 1.

The FAC residues in the squirrel tissues ranged from 182 ppb FAC to 55 864 ppb FAC and were similar to previous findings in coyotes (3). Although the high/low dose ratio was 6.0, the relative FAC levels in like tissues of the low and high dose squirrels varied from 1.5 to 7.45 (Table 1). The reason for the wide range of these ratios is not known. Reliable data on stomach weights were not available and the 1080 absorption could not be calculated. No previous literature citations were located on FAC tissue residues in California ground squirrels.

The liver FAC residues in both the low dose and high dose squirrels were 1.5 to 2.5 times higher than the kidney FAC residues. These ratios are the reverse of those seen in experimental coyotes (3) or canine field cases (4) where the liver/kidney ratios were about 0.5 and 0.4, respectively. Similarly, in an experimental primary 1080 canine poisoning (7), at an oral dose level of 1 mg 1080/kg, the liver FAC and kidney FAC residues were 215 and 442 ppb, respectively (i.e., ratio ~0.5). The reasons for the ratio differences are not known.

Fluoroacetate Residues in Coyote Tissues

The FAC residues in the tissues of secondary 1080-poisoned coyotes, from consumption of 1080-poisoned squirrel carcasses, are given in Table 2.

Kidney, liver, and stomach contents are common submissions for diagnostic analyses. Stomach contents may have higher FAC levels than kidney tissue. However, due to the vomiting, which often occurs in 1080 toxicoses, stomach contents may not be available. In such cases, the gastrointestinal tract may be an appropriate alternative. The data in Tables 1 and 2, along with diagnostic experience (4), indicate that kidney and/or liver are reliable samples for 1080 diagnostic analyses. In 5 of the 6 kidney and liver samples from the 3 coyotes which died of secondary 1080 poisoning, the FAC levels were ≤30 ppb. This further supports the need of a highly sensitive (≤10 ppb) analytical method for detecting FAC residues in secondary poisoning cases. In fact, the residue levels in the kidney and liver of coyote 4 indicate that a 10 ppb FAC detection limit may not be adequate for certain cases of secondary 1080 toxicosis. The liver/kidney FAC ratios in coyotes 5 and 6 were 0.8 and 0.5, respectively. These ratios are similar to those seen in previous coyote and canine toxicoses (3, 4). In field cases of canine 1080 toxicoses the kidney and/or liver FAC residues are commonly less than 50 ppb (5, 7).

The FAC residue findings in these ground squirrels and coyotes, along with diagnostic experiences, indicate that a 1080 diagnostic assay must be reliable at levels of 10 ppb (or less) FAC in tissues. Unfortunately, existing FAC assays (3, 5, 8, 9) are not reliable at this level or require sophisticated techniques and expensive equipment. Future assay developments need to overcome these handicaps to facilitate routine screens for 1080 poisoning.

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DRUG RESIDUES IN ANIMAL TISSUES

Liquid Chromatographic Determination of Monensin in Chicken Tissues with Fluorometric Detection and Confirmation by Gas Chromatography-Mass Spectrometry

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An accurate, sensitive method is described for the determination of monensin residue in chicken tissues by liquid chromatography (LC), in which monensin is derivatized with a fluorescent labeling reagent, 9-anthryldiazomethane (ADAM), to enable fluorometric detection. Samples are extracted with methanol–water (8 + 2), the extract is partitioned between $CHCl_3$ and water, and the $CHCl_3$ layer is cleaned up by silica gel column chromatography. Free monensin, obtained by treatment with phosphate buffer solution (pH 3) at 0°C, is derivatized with ADAM and passed through a disposable silica cartridge. Monensin-ADAM is identified and quantitated by normal phase LC using fluorometric detection. The detection limit is 1 ppb in chicken tissues. Recoveries were 77.6 ± 1.8% at 1 ppm, 56.7 ± 7.1% at 100 ppb, and 46.5 ± 3.7% at 10 ppb fortification levels in chicken. Gas chromatography-mass spectrometry is capable of confirming monensin methyl ester tris trimethylsilyl ether in samples containing residues >5 ppm.

Monensin is a monocarboxylic polyether antibiotic having antimicrobial and anticoccidial activity (1–3). It has been used for the treatment of coccidia in chickens since 1971 (4); and more recently it was found to effectively increase feed efficiency and weight gain in beef cattle (5). Therefore, an analytical method which can detect residual low levels (ppb) of monensin in biological tissues is needed for screening drug residues in animal tissues.

The colorimetric method for determining monensin uses a color reaction with vanillin under acidic conditions (6). This method is only applicable to feeds, premixes, and fermentation broth, and lacks adequate sensitivity for determining traces of monensin in animal tissues.

Today, antibiotics are determined almost exclusively by microbiological methods. These methods are rather lengthy, are not sufficiently sensitive, and lack specificity because of interfering compounds. A thin layer bioautographic method can detect 10 ppb monensin in animal feeds (7) and has been applied to detect residual levels in chicken tissues (8–11). However, its semiquantitative nature, inconsistent results at 10 ppb levels (7), and the relatively tedious analytical procedure limit this method for routine analyses of drug residues in animal tissues.

For these reasons, a new liquid chromatographic (LC) method was developed which uses 9-anthryldiazomethane (ADAM) (12–14) as a fluorescent labeling reagent for the carboxyl group. The preparation of ADAM (14) was modified. Monensin is extracted from the sample, cleaned up, and derivatized with ADAM. The fluorescent ADAM derivative of monensin is identified and quantitated by using LC with fluorometric detection. This method allows the determination of monensin in biological tissues with a high degree of specificity and accuracy at ppb levels. The determination limit is 1 ppb in tissue samples.

Furthermore, a confirmation method with gas chromatography-mass spectrometry (GC-MS) is presented, which may be used to confirm monensin in tissue samples containing residues >5 ppm. In this method, monensin methyl ester tris trimethylsilyl ether is prepared and the mass spectrum is obtained by GC-MS.

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METHOD

Apparatus

(a) *Homogenizer.*—Biotron type BT 10 20 350D (Biotrona Co. Ltd, Switzerland).

(b) *Wrist-action shaker.*—Model-8-1-W (Yayoi Co. Ltd, Tokyo, Japan).

(c) *Chromatographic column.*—Glass, 15 mm id with coarse fritted disk and Teflon stopcock.

(d) *Rotary evaporator.*—Model N-1 (Tokyo Rikakikai Co. Ltd, Tokyo, Japan).

(e) *Tapered reaction vial.*—Amber Reacti-vial, 0.3 mL volume (Pierce Chemical Co., Rockford, IL).

(f) *Liquid chromatograph.*—Model 6000A or 510 solvent delivery system, U6K injector (Waters Associates, Milford, MA).

(g) *Radial compression system.*—Z module (Waters Associates).

(h) *LC column.*—Nova-Pak C_{18} fitted in Z module and μ-Porasil (3.9 mm id × 30 cm) (Waters Associates).

(i) *UV detector.*—Model UVIDEC 100 II UV spectrophotometer (Japan Spectroscopic Co. Ltd, Tokyo, Japan).

(j) *Fluorescence detector.*—Model FP-210 spectrofluorometer (Japan Spectroscopic Co. Ltd).

(k) *Recorder.*—Model YEW 3066 (Yokogawa Electric Works Ltd, Tokyo, Japan).

(l) *Integrator.*—Model C-R3A Chromatopac (Shimadzu Co., Kyoto, Japan).

(m) *Gas chromatograph-mass spectrometer.*—Model JGC-20K gas chromatograph, coupled to Model JMS-D300 mass spectrometer via single stage jet separator, JMA-3100 mass data analysis system (JEOL Ltd, Tokyo, Japan). The system was operated using hard disk-based computer software as supplied. Ionization voltage 70 eV, ionization current 300 μA, ion multiplier 140.

(n) *GC column.*—Glass column of 2 mm id × 50 cm, packed with 2% OV-101 on 80–100 mesh Chromosorb WHP. Column temperature 300°C.

Reagents

(a) *Chemicals.*—Ethyl ether, ethanol, methanol, CH_3CN, CH_2Cl_2, anhydrous Na_2SO_4: pesticide grade (Wako Chemicals Co. Ltd, Osaka, Japan).

(b) *9-Anthraldehyde.*—Technical grade, 90% purity, remainder is anthracene (Aldrich Chemical Co., Milwaukee, WI).

(c) *Hydrazine hydrate.*—Reagent grade (Wako Chemicals Co. Ltd).

(d) *9-Anthraldehyde hydrazone.*—Prepared from 9-anthraldehyde and hydrazine hydrate according to method of Nakaya et al. (12).

(e) *Active MnO_2.*—Prepared from $KMnO_4$ and $MnSO_4$ according to method of Attenburrow et al. (15).

(f) *Saturated ethanol solution of KOH.*—Add ca 1 g KOH to 10 mL ethanol and shake. Use supernate.

(g) *Filter paper.*—Whatman No. 1.

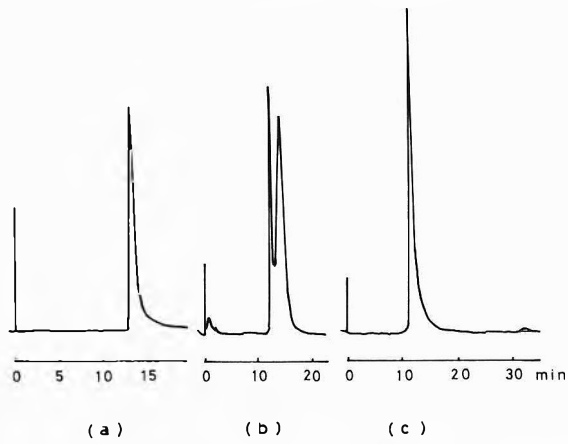

Figure 1. Liquid chromatograms: (a) 9-anthraldehyde hydrazone, 2.42 μg/μL ethyl ether; (b) 2 μL of 15-min reaction mixture at 0°C; (c) 2 μL 9-anthryldiazomethane. Mobile phase CH_3CN–water (1 + 1).

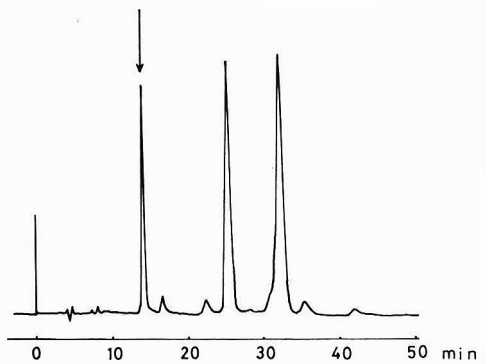

Figure 2. Liquid chromatogram of decomposition products of 9-anthryldiazomethane kept in ethyl ether at room temperature without light protection for 1 day. Arrow indicates 9-anthryldiazomethane. Mobile phase CH_3CN–water (7 + 3).

(h) *Extraction solvent.*—Methanol–water (8 + 2).

(i) *Phosphate buffer.*—Dissolve 13.6 g KH_2PO_4 in 1 L water and add 0.9 g H_3PO_4 (pH ca 3).

(j) *Alumina.*—Neutral, activity 1 (Woelm Pharma GmbH & Co., Eschwege, GFR).

(k) *Silica gel.*—Kieselgel 60, 70–230 mesh, Art. 7734 (E. Merck, Darmstadt, GFR). Dry at 140°C for 3 h and store in desiccator.

(l) *Silica gel cartridge column.*—Sep-Pak silica (Waters Associates, Inc.).

(m) *Monensin sodium.*—Label declaration, 922 μg/mg (American Hoechst Co., La Jolla, CA). (*1*) *Stock solution.*—1000 μg/mL. Dissolve 10.9 mg monensin sodium (or if different label declaration, amount to give 10 mg) in 10 mL methanol. Store in dark, cool place. Discard after 30 days. (*2*) *Standard working solution.*—Dilute stock solution with methanol to 10, 1, and 0.1 μg/mL.

(n) *LC mobile phase.*—CH_3CN–water (1 + 1) and CH_2Cl_2–methanol (19 + 1). Filter before use through microporous filter.

(o) *Microporous filter.*—Millipore type HA (0.45 μm, for water), type FH (0.5 μm, for organic solvent). Fitted to all-glass filter holder, xx15 047 00 (Japan Millipore Ltd, Tokyo, Japan).

(p) *Diazomethane in ethyl ether.*—Prepare from *N*-nitrosomethylurea according to method of Bachman and Struve (16). Caution: Diazomethane is highly toxic carcinogen and reaction could be explosive. Reagent must be prepared fresh daily and reaction must be carried out in fume hood with utmost care.

(q) *Silylating reagent.*—Tri-sil Z (Pierce Chemical Co.).

Preparation of 9-Anthryldiazomethane

Because 9-anthryldiazomethane (ADAM) is unstable when exposed to light at room temperature, all preparation operations must be done as quickly as possible, protecting from light and keeping temperature of reaction mixture at or near 0°C. Remove peroxides from ethyl ether by column chromatography on alumina just before use.

Weigh 242 mg (1.1 mmol) 9-anthraldehyde hydrazone into 200 mL Erlenmeyer flask containing 100 mL ethyl ether. Use amber flask or cover flask with aluminum foil to protect from light. Cool solution to 0°C in ice bath. Add 800 mg active MnO_2 all at once followed by 0.6 mL saturated ethanol solution of KOH. Vigorously stir 30 min, with ice cooling. After reaction, filter reaction mixture through folded paper into ice-cooled 200 mL separatory funnel. Add 20 mL ice-cooled water to same separatory funnel and shake. After setting to separate, discard aqueous phase and drain ethereal phase through anhydrous Na_2SO_4 into 100 mL volumetric flask. Dilute to volume with ethyl ether and transfer contents to 100 mL amber Erlenmeyer flask (or Erlenmeyer flask covered with aluminum foil) and store in freezer at or below −20°C. This solution contains ADAM at concentration of about 10 μmol/mL. Check purity of ADAM with LC using following conditions: column, Nova-Pak C_{18}; eluant, CH_3CN–water (1 + 1); UV detector, set at 254 nm. This solution is ready for use for derivatization of monensin.

Extraction and Cleanup

Weigh 10.0 g chopped sample into 100 mL centrifuge tube. Add 20 mL extraction solvent, methanol–water (8 + 2), and homogenize 10 min. Rinse homogenizer shaft with 3 mL extraction solvent twice and add rinse to centrifuge tube. Shake centrifuge tube 10 min on wrist-action shaker set at fast rate, and centrifuge 10 min at 2000 rpm. Filter supernate through folded paper into 100 mL Erlenmeyer flask. Add 20 mL extraction solvent to residue in centrifuge tube, break up lumps with spatula, and shake 10 min on wrist-action shaker. Centrifuge 10 min at 2000 rpm, filter supernate through folded paper, and combine filtrate with first extract in 100 mL Erlenmeyer flask.

Pour combined extract into 200 mL separatory funnel containing 50 mL $CHCl_3$. After mixing, add 50 mL water and vigorously shake 1 min. After setting to separate, drain $CHCl_3$ phase through folded paper into 200 mL round-bottom flask. Add 30 mL $CHCl_3$ to aqueous phase in separatory funnel and shake again. Set to separate and drain $CHCl_3$ phase through folded paper into previous 200 mL round-bottom flask. Draw off with $CHCl_3$ phase any precipitate at interface. Evaporate $CHCl_3$ from extract to dryness using rotary evaporator at 40–45°C and add 5 mL $CHCl_3$ to dissolve residue.

Dry-pack and tap 5 g silica gel into chromatographic column and add 3 g anhydrous Na_2SO_4 on top. Wash column with 30 mL $CHCl_3$ and discard this wash. Quantitatively transfer above $CHCl_3$ solution to silica gel column, rinse flask twice with 2 mL $CHCl_3$, and add rinse to column. Elute with 50 mL $CHCl_3$ as fraction 1 and discard this fraction. Then elute with 50 mL $CHCl_3$–methanol (19 + 1) as fraction 2 into 100 mL round-bottom flask. Evaporate solvent from fraction 2 with rotary evaporator at 40–45°C and dissolve residue in 2 mL methanol.

Cool 30 mL phosphate buffer solution (pH 3) in 100 mL Erlenmeyer flask with ice. Quantitatively transfer above

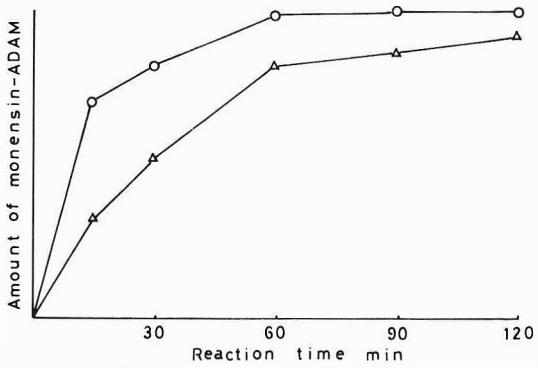

Figure 3. Reaction time vs monensin-ADAM formation at room temperature. O, in methanol–ethyl ether (4 + 1); Δ, in methanol–ethyl ether (2 + 5).

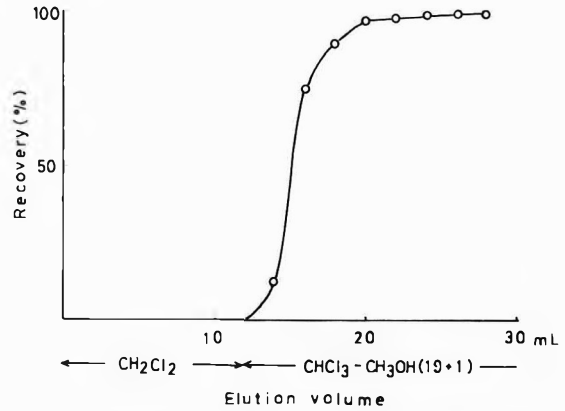

Figure 4. Elution pattern of monensin-ADAM from silica cartridge.

methanol solution to cooled buffer solution in 100 mL Erlenmeyer flask, rinse round-bottom flask twice with 1 mL methanol, and add rinse to buffer solution; maintain ice cooling for 5 min with occasional swirling.

Quantitatively transfer above buffer solution to 100 mL separatory funnel and add 50 mL $CHCl_3$. Shake vigorously 2 min and set to separate. Drain $CHCl_3$ phase into second 100 mL separatory funnel. Add additional 30 mL $CHCl_3$ to aqueous phase in first separatory funnel and shake again. After setting to separate, drain $CHCl_3$ phase into second separatory funnel. Wash combined $CHCl_3$ phase with 5 mL water and drain $CHCl_3$ phase into 100 mL round-bottom flask. Evaporate $CHCl_3$ from extract with rotary evaporator at 40–45°C and dissolve residue in 2 mL methanol.

Derivatization and Cartridge Column Cleanup

Add 0.5 mL ADAM solution of ethyl ether to methanol solution of extract obtained above and let mixture stand overnight at room temperature with complete protection from light. Evaporate solvent completely from reaction mixture, using rotary evaporator at 40°C and redissolve residue in 2 mL CH_2Cl_2.

Attach silica cartridge to 2 mL syringe barrel. Quantitatively transfer above CH_2Cl_2 solution to syringe and force solvent gently through column by applying pressure on syringe plunger.

Rinse flask with 2 mL CH_2Cl_2 and add rinse to cartridge and elute. Wash cartridge with additional 6 mL CH_2Cl_2 in 2 mL portions and discard CH_2Cl_2 eluate. Elute ADAM derivative of monensin with 14 mL $CHCl_3$–methanol (19 + 1) in 2 mL portions into 30 mL pear-shape flask. Evaporate solvent with rotary evaporator and redissolve residue in 1 mL $CHCl_3$. Reserve this as sample solution for injection into LC apparatus.

Liquid Chromatography and Quantitation

Equilibrate μ-Porasil column 30–45 min with mobile phase, CH_2Cl_2–methanol (19 + 1), flow rate at 1.0 mL/min. Set fluorescence detector excitation wavelength at 365 nm and emission at 412 nm.

Prepare standard solutions of monensin-ADAM as follows: Cool 3 sets of 30 mL phosphate buffer solution (pH 3) in 100 mL Erlenmeyer flasks with ice. Add 1, 2, and 3 mL working standard solution of 0.1 μg monensin sodium/mL to each flask. Hold 5 min in ice bath with occasional swirling. Extract phosphate buffer solution with 50 and 30 mL portions of $CHCl_3$, wash $CHCl_3$ extract with 5 mL water, and evaporate using 30 mL pear-shape flask with rotary evaporator at 40°C. Dissolve residue in 2 mL methanol and add 0.5 mL ADAM solution in ethyl ether. Hold at room temperature overnight with complete light protection. Clean up with disposable silica cartridge as described before and obtain 1 mL $CHCl_3$ solutions of monensin-ADAM standard, which correspond to 0.1, 0.2, and 0.3 μg/mL of monensin sodium. Construct calibration curve by plotting fluorometric response (peak height or peak area of integrator) vs amount of standard.

Inject sample solutions interspersed with standard solutions of monensin-ADAM to ensure accurate identification of monensin-ADAM in sample solution. If monensin-ADAM is identified in sample solution, verify by co-injection of standard solution and sample solution. Calculate amount of monensin sodium in sample solution by comparing peak height or peak area with calibration curve. Calculate concentration of monensin sodium in sample by dividing amount of monensin sodium in sample solution obtained above by sample weight (10.0 g).

Mass Spectrometric Confirmation

If >5 ppm residual monensin sodium is detected in sample, confirm monensin with gas chromatography-mass spectrometry.

Extract monensin sodium from sample, and clean up by partition between $CHCl_3$ and water, followed by silica gel column chromatography. Transform to free monensin (—COOH form) by treatment with phosphate buffer solution, as described before.

Prepare ethyl ether solution of diazomethane (15). Esterify free monensin by reaction with diazomethane in ethyl ether at room temperature for 30 min. Evaporate ethyl ether from reaction mixture, using 30 mL pear-shape flask, by rotary evaporator at 40°C. Dissolve residue in 0.3 mL CH_2Cl_2 and transfer to 0.3 mL amber Reacti-vial. Evaporate CH_2Cl_2 under mild stream of nitrogen on water bath at 35–40°C. Add 0.2 mL Tri-sil Z to Reacti-vial, stopper, and heat at 80°C for 30 min on heating block. This solution is ready for injection into GC-MS apparatus. Complete analysis on same day as derivatization.

Results and Discussion

Liquid chromatography offers several advantages for determination of nonvolatile drugs, but its application to monensin was hindered by the lack of a suitable means of detection. Monensin has no strong chromophore and has only weak absorption in the UV region, which is not well suited for residual detection. The formation of a detectable derivative is a useful approach to this type of problem and the fluorescent derivative was thought to be adequate for detection at the ppb level. Various fluorescent labeling reagents

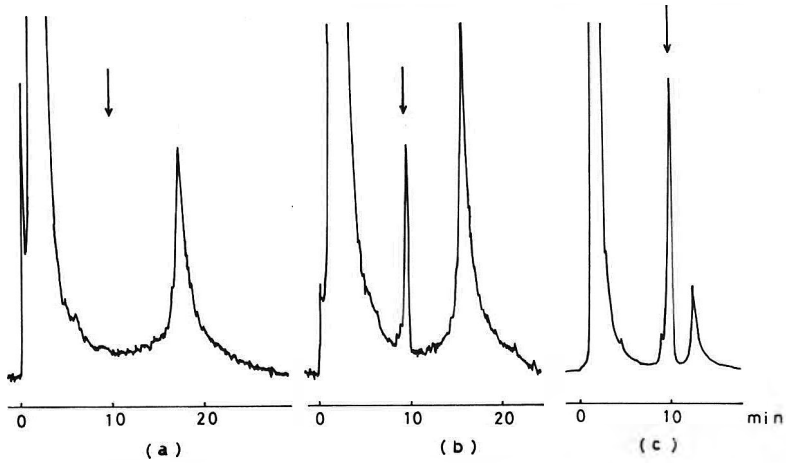

Figure 5. Liquid chromatograms of chicken extract: (a) blank chicken tissue extract; (b) chicken tissue spiked with 10 ppb concentration of monensin sodium; (c) chicken tissue spiked with 100 ppb concentration of monensin sodium. Arrow indicates monensin-ADAM. Mobile phase CH_2Cl_2–methanol (19 + 1).

were reported for carboxyl and hydroxyl groups (17) and we selected 9-anthryldiazomethane (ADAM) because of its high reactivity, mild reaction conditions, smooth reaction with the carboxylic group, easy experimental operation, and high fluorescence level.

Nakaya et al. first reported the synthesis of ADAM in 1967 (12), and later Barker et al. reported a simpler and more convenient synthetic method (14). This reagent was used to derivatize fatty acids (13) and prostaglandins (18). In Japan, ADAM is now obtainable from a commercial source, but its purity is not very good. Hence we synthesized ADAM according to the procedure of Barker et al. (14), but with following modifications: (*1*) Ethyl ether used for the oxidation reaction was purified before use to remove peroxides. (*2*) Reaction temperature was decreased to 0°C, and reaction time was determined to be 30 min. (*3*) After MnO_2 was filtered off, the reaction mixture was washed with water and dried over Na_2SO_4. (*4*) ADAM was stored as an ethereal solution at −20°C. These modifications somewhat avoided formation of by-products, and the reaction time was determined by LC check (Figure 1).

Evaporation of reaction solvent (ethyl ether) under reduced pressure gave ADAM as a dark red crystalline product which was almost pure by LC check. About half of this crystalline product decomposed after storage at −20°C for a month. In ethyl ether solution at −20°C, we observed that ADAM can be stored in darkness with no significant decomposition for periods exceeding 5 months. This solution can be used directly for derivatization. The concentration of ADAM was estimated from 90% yield of the oxidation reaction. When kept in ethyl ether at room temperature without light protection, a considerable portion of ADAM decomposed, as shown in Figure 2.

Synthesized ADAM was reacted with acetic acid and stearic acid to check its reactivity and to obtain reference compounds for LC elution conditions. Acetic acid reacted almost spontaneously and stearic acid completely reacted within 5–10 min at room temperature. The products were identified with GC-MS (M^+ 250 for ADAM-acetate, M^+ 474 for ADAM-stearate).

The reactivity of monensin to ADAM reagent was checked. Monensin usually exists as a sodium salt which forms a stable, lipophilic complex (3). But in order to react with ADAM, monensin sodium must be converted to free monensin (acid form) (—COO^- NA^+ → —COOH). Because free monensin (acid form) is unstable in acidic conditions and degrades rapidly (1), milder conditions were needed.

First, free monensin was obtained by elution of monensin sodium with methanol through an ion exchange column (Bio-Rex 70, —COOH form). But this is rather tedious for routine analyses, so treatment with phosphate buffer solution was attempted. It was found that dissolution of monensin sodium into ice-cooled 0.1M KH_2PO_4 solution (pH ca 4.5) and extraction with $CHCl_3$ gave free monensin quantitatively. However, in the presence of sample extracts, 0.1M KH_2PO_4 solution acidified to pH 3 with phosphoric acid gave higher yields and this condition was used.

Reaction of monensin with ADAM was relatively slow in nonpolar solvent. In ethyl ether, this reaction proceeds very slowly; Figure 3 shows the reaction rate in ethyl ether–methanol (5 + 2) and in ethyl ether–methanol (1 + 4) at room temperature. Reaction in the latter solvent mixture is faster

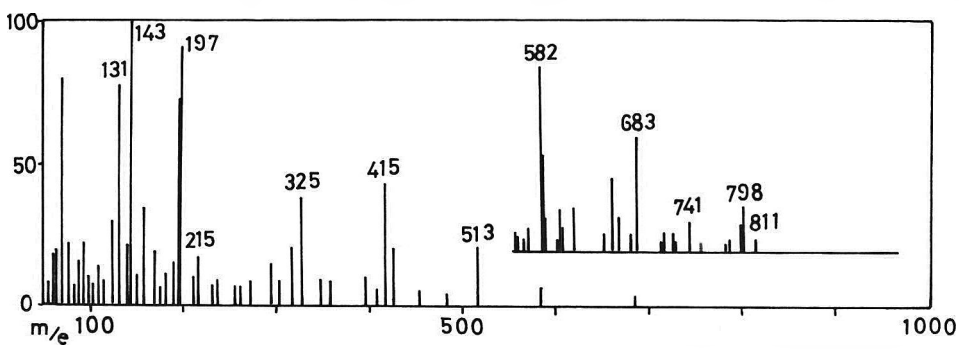

Figure 6. Electron impact mass spectrum of monensin methyl ester tris trimethylsilyl ether.

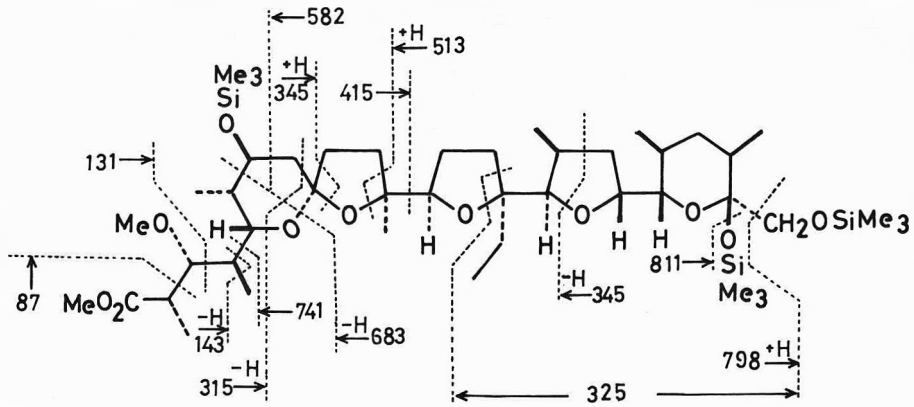

Figure 7. Tentatively assigned fission pattern of monensin methyl ester tris trimethylsilyl ether in electron impact mass spectrum.

and is complete after 1 h. The reaction rate in the presence of more methanol was almost the same. Pure monensin reacted with ADAM completely after 1 h, but in the presence of sample extracts, the reaction rate was somewhat slow and overnight reaction was used.

The extraction solvent was the same as described in the literature, (7, 11) but the cleanup procedure was modified to include $CHCl_3$–water partition before silica gel column chromatography. The extracted material from 10 g chicken tissue was about 600–700 mg and was not completely soluble in $CHCl_3$, so it was difficult to chromatograph this residue on a small scale column. Partition of sample extract between $CHCl_3$ and water reduced the amount of extracted residue to about 20–30 mg. This was completely dissolved in $CHCl_3$ and easily chromatographed on 5 g silica gel. Without silica gel column chromatography, extracted material from chicken tissue formed an emulsion when treated with phosphate buffer solution.

The reaction mixture of monensin and ADAM can be directly injected into the LC apparatus if concentrations of monensin in the sample exceed 1 ppm. Below this concentration, a cleanup process is necessary for removing excess ADAM and by-products. Figure 4 shows the elution pattern of monensin-ADAM from a Sep-Pak silica cartridge. This is a convenient, fast, and solvent-saving cleanup procedure.

Reverse phase LC conditions were attempted first but the peak of monensin-ADAM was close to the peaks of ADAM-acetate, ADAM-stearate, and decomposition products of ADAM. Good separation was obtained using normal phase conditions such as a μ-Porasil column. Figure 5 shows liquid chromatograms of chicken tissue extract blank and fortified sample extracts. The peak of monensin-ADAM was clearly identified and no interferences were observed. The excitation and emission wavelengths of fluorometric detection were selected as described in the literature (13).

The calibration curve is constructed after transformation of monensin sodium in a working standard solution to free monensin, reaction with ADAM, and cleanup on a silica cartridge. The calibration curve is linear and passes through zero.

Average recoveries of monensin sodium added to blank chicken tissues were determined as follows: Chicken tissues had been previously analyzed for monensin sodium with this proposed method, and tissue, in which monensin was not detected, was used as a blank sample. Blank samples spiked at 1 ppm and 100 and 10 ppb monensin sodium, each level in triplicate, were analyzed with this proposed method and average recoveries and CV % were as follows: 77.6 ± 1.8% at 1 ppm, 56.7 ± 7.1% at 100 ppb, and 46.5 ± 3.7% at 10 ppb fortification levels. The recovery at 1 ppm level was satisfactory, but recoveries at 100 and 10 ppb levels were not. The coefficients of variation were sufficiently small.

The detection limit was determined using blank chicken tissue spiked at various levels; chicken tissue spiked at 1 ppb concentration gave a clearly identifiable peak of monensin-ADAM, its peak height was about 5 times the noise level. This method could be also applied to chicken liver and beef. Samples obtained from commercial sources of chicken tissue, chicken liver, and beef were analyzed and monensin was not detected. This proposed method is applicable to routine analyses of residual monensin in animal tissues.

A gas chromatographic-mass spectrometric method is used for confirmation of monensin. Monensin-ADAM was difficult to elute from the gas chromatographic column after silylation, so monensin methyl ester tris trimethylsilyl ether was prepared for GC-MS analysis. This compound gave a relatively symmetrical peak and an identifiable mass spectrum on electron impact of 70 eV (Figure 6). Figure 7 shows the tentatively assigned fission patterns. These furnish reliable data for confirmation of monensin in the sample. The detection levels of this GC-MS method were determined using blank samples spiked at various concentrations of monensin sodium. From blank samples spiked at 5 ppm, we could observe a definitely identifiable peak of monensin methyl ester tris trimethylsilyl ether in total ion monitoring of GC-MS and we could obtain a clear mass spectrum. This detection limit may be varied by the GC-MS apparatus used; if an increased sample amount is used for extraction, we may be able to identify monensin in samples of concentrations <5 ppm.

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Determination of Ampicillin Residues in Fish Tissues by Liquid Chromatography

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A liquid chromatographic (LC) method is described for determination of ampicillin residues in fish tissues. The drug is extracted from tissues with methanol, and the extract is evaporated to dryness. This residue is cleaned up by Florisil cartridge chromatography. LC analysis is carried out on a Nucleosil C18 column, and ampicillin is quantitated by ultraviolet detection at 222 nm. Recoveries of ampicillin added to tissues at levels of 0.2 and 0.1 ppm were 73.2 and 61.5%, respectively. The detection limit was 3 ng for ampicillin standard, and 0.03 ppm in tissues.

Ampicillin, 6-(2-amino-2-phenylacetamido) penicillanic acid, is used to protect cultured fish against a wide variety of both Gram-negative and Gram-positive bacteria (1). The presence of drug residues in tissues of food-producing animals is undesirable from a public safety standpoint, so it is necessary to have available a sensitive method to determine ampicillin residues in tissues.

Many methods for the quantitative determination of ampicillin and ampicillin derivates have been described. Ampicillin derivates are determined spectrophotometrically (2, 3), spectrofluorometrically (4–6), and potentiometrically (7) after conversion to penicillanic and/or penicilloic acid by treatment with either alkali or acid in the presence of copper or mercury ions. These procedures are based on cleavage of the β-lactam ring to the corresponding penicillanic or penicilloic acid, so ampicillin is not determined specifically.

Microbiological determinations of ampicillin in capsules (8), in body fluids (9, 10), in milk (11), and in tissues (12) have been reported. Such methods are lengthy for one sample. A thin layer chromatographic method, using bioautographic detection, has been reported for determining ampicillin in beef tissues (13). This method is sensitive (tissues were fortified at 0.01 ppm), but is only semiquantitative and requires considerable time and labor.

Liquid chromatographic (LC) methods have been adapted to the determination of ampicillin in capsules (14, 15) and in body fluids fortified at levels between 0.5 and 1000 ppm (16). These methods are not sufficiently sensitive to monitor residual amounts of ampicillin in tissues. An LC post-column reaction system was applied to ampicillin in body fluids (17). This method, which measures the reaction product of ampicillin, is sensitive but is not suitable for daily analyses.

The present paper describes a simple, sensitive method for determination of ampicillin in fish tissues by LC at levels as low as 0.03 ppm.

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METHOD

Reagents

Use analytical reagent grade chemicals and deionized water unless otherwise specified.

(a) *Solvents.*—Methanol, acetonitrile, ethyl ether, and *n*-propyl alcohol (Wako Pure Chemical Industry Ltd, Osaka, Japan).

(b) *Disodium hydrogen phosphate.*—Waco Pure Chemical Industry Ltd.

(c) *Citric acid.*—Waco Pure Chemical Industry Ltd.

(d) *Buffer solution (pH 6.0).*—Mix 0.02M Na_2HPO_4 and 0.01M citric acid solutions and adjust to pH 6.0.

(e) *LC elution solvent.*—Methanol–buffer solution (pH 6.0) **(d)** (15 + 85).

(f) *Adsorbents.*—Sep-Pak Florisil cartridge (Waters Associates, Inc.). Attach cartridge to 10 mL glass syringe. Prewash each cartridge with 5 mL methanol and apply air pressure to column until traces of moisture disappear. Then wash with 5 mL ethyl ether and apply air pressure as before. Dry in column oven for 10 min at 30°C.

(g) *Ampicillin standard solution.*—Prepare stock solution at 100 μg/mL, using 10 mg ampicillin (Sigma Chemical) in 100 mL buffer solution **(d)**. Store at 10°C in the dark. Prepare 1 μg/mL working standard solution in buffer solution **(d)**, using 1 mL stock solution. Prepare daily.

Apparatus

(a) *Liquid chromatograph.*—Shimadzu LC-3A equipped with Shimadzu SPD-2A ultraviolet spectrometer and Shimadzu CTO-2A column oven. Chromatographic conditions: flow rate, 1 mL/min; temperature, 30°C; detection, 222 nm.

(b) *Chromatographic column.*—Nucleosil C18, stainless steel, 150 mm × 4.6 mm id (Gaskuro Kogyo, Inc.).

(c) *High-speed homogenizer.*—Ultra-Turrax T18 (Janke & Kunkel GmbH & Co., Switzerland).

(d) *Centrifuge.*—Model H-100-F (Kokusan Enshinki Co., Ltd, Tokyo, Japan).

Extraction and Cleanup

Accurately weigh 10 g minced tissue, homogenize 3 min at maximum speed with 50 mL methanol, centrifuge 10 min at 3000 rpm, and filter through cotton. Homogenize residue with another 50 mL methanol, centrifuge, and filter as before. Combine filtrate in flask and add 20 mL *n*-propyl alcohol. Evaporate to dryness under vacuum or rotary evaporator at 40°C. Rinse flask with 5 mL ethyl ether and apply rinse to

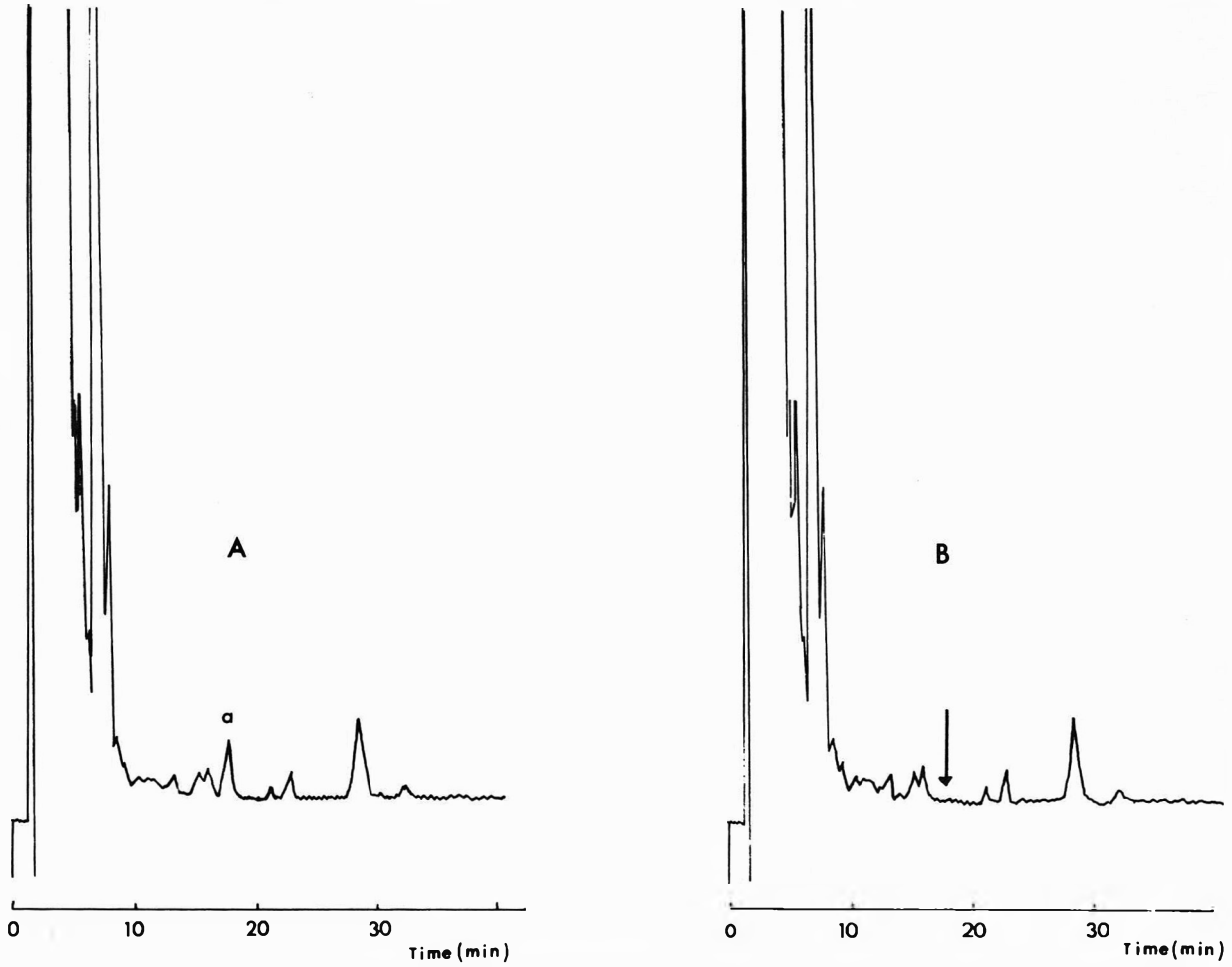

Figure 1. Typical chromatogram of spiked and unspiked commercial yellow tail tissue: A, 2 μg ampicillin in 10 g tissue (a = 20 ng ampicillin); B, unspiked tissue (arrow = retention time of ampicillin).

prewashed Florisil cartridge; discard eluate. Rinse flask with 5 mL ethyl ether–acetonitrile (1 + 9) solution and apply rinse to cartridge; discard eluate. Dissolve residue in flask with 5 mL acetonitrile–water (7 + 3) solution and apply to cartridge. Rinse flask with 2 mL acetonitrile–water (7 + 3) and apply rinse to cartridge. Collect eluates and evaporate to dryness under vacuum on rotary evaporator at 40°C. Dissolve residue in 1 mL buffer solution (**d**). Filter through 0.45 μm micropore filter and apply to LC apparatus.

Analysis and Calculation

Inject 10 μL working standard and sample solutions into LC system and measure peak heights, respectively. Calculate concentration by following formula:

$$\text{Ampicillin, ppm} = (R/R') \times C' \times 0.1$$

where R' and R = peak height for working standard and sample solutions injected, respectively; and C' = concentration of ampicillin in working standard, μg/mL.

Results and Discussion

The stability of ampicillin in various buffer solutions with pH 3.0–7.0 has been examined. Although solutions of 100 μg/mL of ampicillin at pH 6–7 stored at 10°C were stable for 2 weeks or more, 10 μg/mL ampicillin solutions were slightly unstable even at that pH range, as shown in Table 1.

The optimal LC operating conditions were studied by varying the mobile phase composition, column temperature, and flow rate. Variation of pH of mobile phase resulted in considerable changes in k' (capacity factor) of ampicillin, in stability and in noise of baseline. Stability and noiseless baseline were obtained when pH of mobile phase was in the range of 6–7. The sensitivity of detection of ampicillin in the mobile phase increased rapidly below 250 nm. Detection at 222 nm afforded the best compromise between noise and sensitivity. Under the conditions selected for detection, as shown in *Method,* ampicillin was well separated from other interfering components. Ten injections of 100 ng ampicillin each gave an average retention time of 17.59 min (range 17.56–17.64 min) with a standard deviation of 0.023 min and a coefficient of variation of 0.13%. The constant retention time for ampicillin is an index of its identity.

A calibration curve was liner over the range 3–150 ng ampicillin.

Ampicillin was extracted by methanol and extracts were evaporated to dryness with addition of *n*-propyl alcohol to inhibit bumping. The Florisil column was used to remove other co-extractives that might irreversibly adsorb on the LC analytical column and interfere in the measurement of ampicillin on the chromatogram. Ampicillin was irreversibly adsorbed on the activated Florisil, so Florisil was inactivated with methanol. Interferences were removed with 5 mL ethyl ether followed by 5 mL ethyl ether–acetonitrile (1 + 9) solution. Ampicillin was eluted from the column with 5 mL acetonitrile–water (7 + 3) solution. Figure 1 shows typical chromatograms for samples of commercial yellow tail tissues, unspiked and spiked at 0.2 ppm, respectively.

Table 1. Degradation of ampicillin in various pH solutions

	Recovery, %[b]									
	pH 3		pH 4		pH 5		pH 6		pH 7	
Days[a]	100	10	100	10	100	10	100	10	100	10
1	95	95	100	99	100	100	100	100	100	99
2	90	85	98	95	100	100	100	100	98	95
3	83	80	96	94	100	99	100	99	96	94
7	68	66	95	89	100	91	100	93	95	89
14	54	53	90	86	100	91	100	93	90	86
18	46	42	87	86	100	91	100	93	87	86

[a]Time stored at 10°C.
[b]Compared with initial concentration, μg/mL.

Table 2. Recovery of ampicillin added to 10 g portions of yellow tail tissues

Added, μg	Found, μg	Rec., %
2.0	1.552	77.6
	1.516	75.8
	1.410	70.5
	1.472	73.6
	1.370	68.5
Av.		73.2
SD		3.73
CV, %		5.09
1.0	0.557	55.7
	0.644	64.4
	0.628	62.8
	0.691	69.1
	0.557	55.7
Av.		61.5
SD		5.81
CV, %		9.44

Recovery studies were performed by adding 2.0 and 1.0 μg ampicillin to 10 g minced yellow tail tissues. These recoveries (Table 2) were satisfactory for analysis. The utility of the method was demonstrated by its application to commercial yellow tail tissues. No ampicillin was detected in 20 commercial tissues by the present method. The detection limit, determined as 3 times noise level, was 3 ng, which corresponds to 0.03 ppm ampicillin in tissue. Other drugs which are normally used to protect cultured fishes, such as tetracyclines, sulfonamides, nalidixic acid, oxolinic acid, and piromidic acid did not interfere.

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PESTICIDE AND INDUSTRIAL CHEMICAL RESIDUES

Determination of Halogenated Contaminants in Human Adipose Tissue

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A method has been developed for determination of organochlorine contaminants in human adipose tissue. After fat extraction from the tissue with acetone–hexane (15 + 85, v/v), organochlorines were fractionated from fat by gel permeation chromatography with methylene chloride–cyclohexane (1 + 1, v/v) as solvent. After Florisil column cleanup, the GPC extract was analyzed by capillary column gas chromatography using 2 columns of different polarity. Compound identity was confirmed by gas chromatography–mass spectrometry using selected ion monitoring. Recoveries for fortification levels of 10–500 ng/g were greater than 80% except for trichlorobenzene and hexachlorobutadiene (ca 60%).

Because of their persistence and potential for bioaccumulation, a need exists to determine the levels of organochlorine (OC) contaminants in human adipose tissue as an indicator of exposure and to assist in human health risk assessment. Consequently, surveys have been conducted to determine the presence of the more common organochlorine pesticides and other pollutants such as polychlorinated biphenyls in adipose tissue (1–4). The methods used in these surveys for the isolation of OC contaminants from human adipose tissue are relatively tedious, labor-intensive, and not readily amenable to automation. However, gel permeation chromatography (GPC) has been shown to be effective for separation of organic compounds from a variety of fat matrices (2, 5–10) and, moreover, automated GPC equipment is available for fractionation of large numbers of fat extracts (5).

Recently, we reported a GPC method for analysis of organophosphate triesters in human adipose tissue (2). This method demonstrated potential for multiclass, multiresidue determination of contaminants in human adipose tissue; however, when applied to OC contaminants, the solvent system used was not effective in isolating the target compounds into a narrow elution band. Tessari et al. (6) used GPC with 15% methylene chloride–cyclohexane as solvent, and MacLeod et al. (8) used cyclohexane as solvent for OC determination in human adipose tissue; they demonstrated GPC to be an effective cleanup procedure. However, their methods also gave a wide elution band for the OC fraction. A number of workers have used GPC, with methylene chloride–cyclohexane (1 + 1, v/v) as solvent, to isolate OC contaminants from various fat matrices (5, 7). Therefore, this solvent system was evaluated for effective isolation of OC contaminants from human adipose tissue. The effectiveness of dual capillary column gas chromatography (GC) and gas chromatography–mass spectrometry (GC-MS) has also been evaluated for identification and quantitation of OC contaminants.

METHOD

Reagents

(a) *Solvents.*—Acetone, hexane, cyclohexane, and methylene chloride (Caledon Laboratories, Georgetown, Ontario); all distilled-in-glass grade.

(b) *Glass wool and purified water.*—Prepare as previously described (9).

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(c) *Anhydrous sodium sulfate.*—Reagent grade, granular. Wash with acetone, hexane, and methylene chloride. Remove solvent by flushing with Florisil-scrubbed (see (f) for Florisil preparation) purified nitrogen and heat at 700°C overnight. Cool and store in clean jar with Teflon-lined cap.

(d) *Standard solutions.*—Pesticide standards were obtained from the Pesticides Section, Food Directorate, Health and Welfare Canada. Other standards were obtained from commercial sources. All standards were 95 + % pure. Prepare 500 ng/μL stock solutions and appropriate working mixtures as required.

(e) *Gel beads.*—Bio-Beads S-X3 (Bio-Rad Laboratories, Mississauga, Ontario), porous styrene-divinyl benzene copolymer, 200–400 mesh.

(f) *Florisil.*—PR grade (Mandel Scientific, Rockport, Ontario). Wash with methylene chloride until free of interferences. Remove solvent by flushing with Florisil-scrubbed purified nitrogen. Activate at 275°C overnight, let cool, and deactivate with 2% (w/w) purified water (previously extracted with hexane). Store in clean glass jars with Teflon-lined caps.

Apparatus

(a) *Manual GPC apparatus.*—As previously described (2) except column and solvent system as described in (b).

(b) *Automated GPC system.*—Autoprep gel permeation chromatograph (Analytical Biochemistry Laboratories, Inc., PO Box 1097, Columbia, MO 65205) Model 1002A, with 60 g Bio-Beads S-X3 resin, 200–400 mesh, in 60 × 2.5 cm id column compressed to ca 48 cm bed length. Elution solvent methylene chloride–cyclohexane (1 + 1, v/v), flow rate calibrated to 5.0 mL/min, operating pressure 7–10 psi. Sampling valve was replaced with Rheodyne Model 5020 valve with 1/16 in. tubing, permitting 0.5 mL reduction of void volume to sampling loops.

(c) *Extraction apparatus.*—Tekmar SDT series overhead Tissumizer with SDT-182 EN shaft and generator for use in water and/or organic solvent medium.

(d) *Centrifuge.*—Table-top, IEC Model HNSII. Operate at 1600 rpm.

(e) *Chromatography column.*—Chromaflex column, 6 mm id, with 50 mL solvent reservoir (Kontes, No. K-420100-0021).

(f) *Gas chromatograph.*—Varian Model 4600 equipped with ^{63}Ni electron capture detector and interfaced to Vista 402 chromatography data system with dual disk drives. Equipped with Varian 8000 automatic injector system that injected 1.5 μL aliquots. Column parameters and operating conditions: *(i)* 15 m × 0.25 mm id DB-17 (J & W) fused silica capillary column; oven temperature: initial 80°C, hold 2 min, program at 20°/min to 220°C, hold 1 min, then program 5°/min to 280°C, hold 6 min; helium carrier gas 1.5 mL/min (52 cm/s linear velocity) with nitrogen make-up gas at 30 mL/min; injector 260°C; detector 325°C. *(ii)* 15 m × 0.25 mm id DB-5 (J & W) fused silica capillary column; oven temperature: initial 80°C, hold 2 min, program at 20°/min to 220°C, hold 1 min, then program 5°/min to 275°C, hold 5 min; helium carrier gas 1

Table 1. GPC, Florisil, GC, and GC-MS data for some organochlorine contaminants

Organochlorine	GPC elution vol.,[a] mL	Florisil eluate fraction	MDL[b]	GC retention time[c] DB-17	GC retention time[c] DB-5	GC/MS Selected ions	
1,4-Dichloro Bz (1)	200–200	A	—	2.55	2.79	145.95	148.00
1,3-Dichloro Bz (2)	210–220	A	—	2.69	2.80	145.95	148.00
1,2-Dichloro Bz (3)	210–220	A	—	3.05	3.12	145.95	148.00
1,3,5-Trichloro Bz (4)	200–220	A	11.0	3.73	4.10	179.90	181.90
HCBD (5)	170–210	A	1.2	4.38	4.84	224.85	226.75
1,2,3-Trichloro Bz (6)	210–230	A	5.9	4.86	4.82	179.90	181.90
2,4,5-Trichlorotoluene (7)	200–220	A	14.3	5.32	5.49	179.90	181.90
1,2,3,5-Tetrachloro Bz (8)	200–220	A	13.1	5.36	5.74	213.80	215.85
1,2,3,4-Tetrachloro Bz (9)	200–230	A	4.8	6.18	6.12	213.80	215.80
Pentachloro Bz (10)	200–230	A	1.9	7.09	7.09	247.90	249.80
Hexachloro Bz (11)	200–230	A	1.4	8.30	8.26	283.80	285.80
α-BHC (12)	190–230	B	1.2	8.54	8.17	216.90	218.90
Chlordene (13)	170–210	A	1.2	8.85	8.79	—	—
γ-BHC (14)	200–230	B	1.4	9.04	8.56	216.90	218.90
β-BHC (15)	240–280	B	3.0	9.16	8.50	216.90	218.90
Heptachlor (16)	170–210	A	1.4	9.49	9.50	269.80	271.70
Aldrin (17)	170–210	A	1.2	10.00	10.00	79.05	262.80
Octachlorostyrene (18)	170–210	A	1.1	10.52	10.55	307.85	379.75
Oxychlordane (19)	170–200	B	1.2	10.73	10.65	386.75	388.90
Heptachlor epoxide (20)	170–210	B	1.1	11.00	10.65	352.90	354.90
γ-Chlordane (21)	180–210	B	1.3	11.32	11.06	236.95	374.80
Trans-nonachlor (22)	170–210	A+B	1.3	11.38	11.44	406.85	408.85
α-Chlordane (23)	170–220	B	1.0	11.64	11.29	236.95	374.80
α-Endosulfan (24)	170–220	B	1.2	11.69	11.33	194.90	206.85
o,p'-DDE (25)	170–210	A+B	2.6	11.70	11.15	247.90	317.90
p,p'-DDE (26)	180–210	A	1.2	12.24	11.73	247.90	317.90
Dieldrin (27)	180–210	B	0.9	12.34	11.79	276.85	278.85
Endrin (28)	170–210	B	2.4	13.18	12.26	316.95	318.95
Cis-nonachlor (29)	180–230	B	1.3	13.26	12.72	406.85	408.85
p,p'-DDD (30)	190–220	B	2.1	13.56	12.63	235.05	237.05
o,p'-DDT (31)		A+B	4.0	13.56	12.68	235.05	237.05
p,p'-DDT (32)	180–210	B	1.7	14.32	13.45	235.05	237.05
Photomirex (33)	180–210	A	1.9	14.80	14.18	236.85	271.75
Mirex (34)	180–210	A	1.8	16.73	15.85	236.85	271.75
Methoxychlor (35)	190–210	B	13.5	16.94	14.90	238.15	274.00
Decachlorobiphenyl (36)	170–210	A	—	20.55	19.60	497.65	499.65
Hexabromobiphenyl (37)	200–220	A	6.6	23.20	20.50	467.75	469.55
			—	—	—	627.55	629.45
Aroclor 1260 (38)	180–220	A	—	—	—	291.95	325.85
			—	—	—	359.95	393.80

[a]236 mL bed volume S-X3 gel with methylene chloride–cyclohexane (1 + 1, v/v) eluant.
[b]Minimum detection limit (ng/g) based on area reject of 3000 counts for 1 g sample in 2 mL extract.
[c]Relative to aldrin (RRT = 10.00): retention time 10.41 min (DB-17); 10.90 min (DB-5).

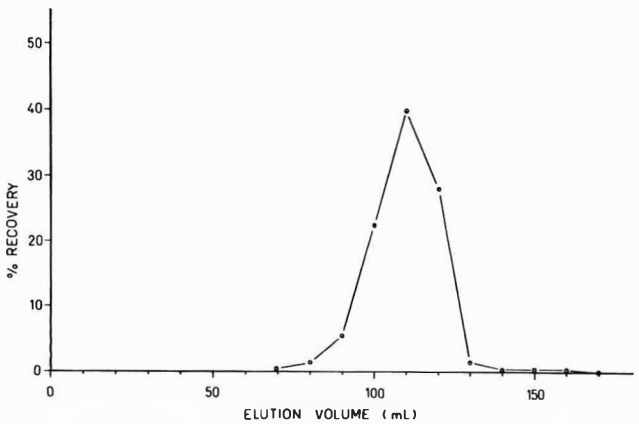

Figure 1. GPC elution profile of human adipose fat with 50% methylene chloride–cyclohexane eluant on Bio-Beads S-X3 (see text).

mL/min (42 cm/s linear velocity) with nitrogen make-up gas at 30 mL/min; injector 260°C; detector 325°C.

(g) *Gas chromatograph–mass spectrometer (GC-MS).*— Hewlett-Packard Model 5992B GC-MS coupled with Model 9825A on-line data system and 2 Model 9885S disc drives. Column parameters and operating conditions: 15 m × 0.25 mm id DB-17 (J & W) fused silica capillary column; oven temperature: initial 116°C, hold 1 min, program at 16°C/min to 276°C, hold 9 min; helium carrier gas 1 mL/min; 2 μL splitless injections were used with splitter opened after 60 s; injector 240°C. MS instrument was operated in selected ion mode (SIM) with dwell times of 40 ms/ion. Glass-lined open split interface/restrictor connected GC column to MS instrument with flow rate of 0.8 mL/min entering spectrometer.

For analysis of hexabromobiphenyl, operating conditions were as follows: oven temperature 200°C, hold 3 min, program at 16°/min to 280°C, hold 12 min; helium carrier gas 1.5 mL/min; injector 300°C.

Extraction of Human Adipose Tissue

Let deep-frozen (−20°C) tissue, obtained from cadavers at autopsy and stored in clean glass vials, thaw overnight in cold room (4°C) and bring to room temperature ca 30 min before extraction. Cut tissue sample in small portions on piece of acetone-rinsed heavy aluminum foil and accurately weigh tissue into tall heavy-wall beaker. Add acetone–hexane (15 + 85, v/v) solvent mixture, 5 mL/g of tissue sample, and precleaned anhydrous sodium sulfate, ca 0.5 g/g of tissue sample. Homogenize mixture with Tissumizer at moderate speed for ca 2 min. (Note: Connective tissues may block homogenizer blades; carefully control speed to avoid splashing.) Rinse homogenizer shaft with ca 20 mL solvent and collect all rinsings. Centrifuge extract at 1600 rpm ca 15 min and filter clear solution through anhydrous sodium sulfate into preweighed round-bottom flask. Rinse filter twice with ca 5 mL extraction solvent. Combine filtrates and remove

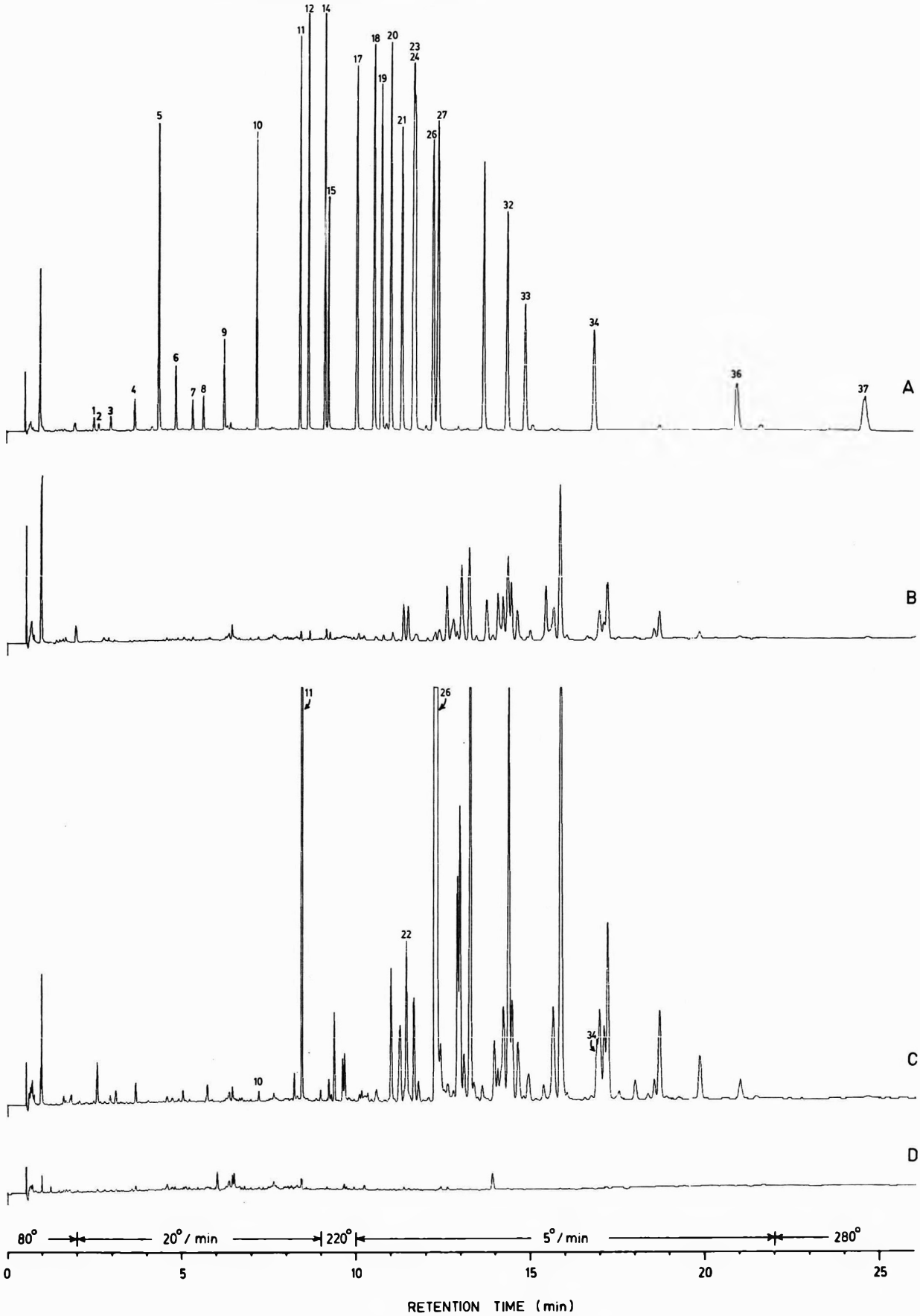

Figure 2. Electron capture GC chromatogram on DB-17 capillary column of (A) OC mixture solution at 50 pg/μL; (B) Arocior 1260 solution at 250 pg/μL; (C) Florisil Fraction A extract of Kingston composite sample; (D) Florisil Fraction A of method blank. (See Table 1 for peak identification.)

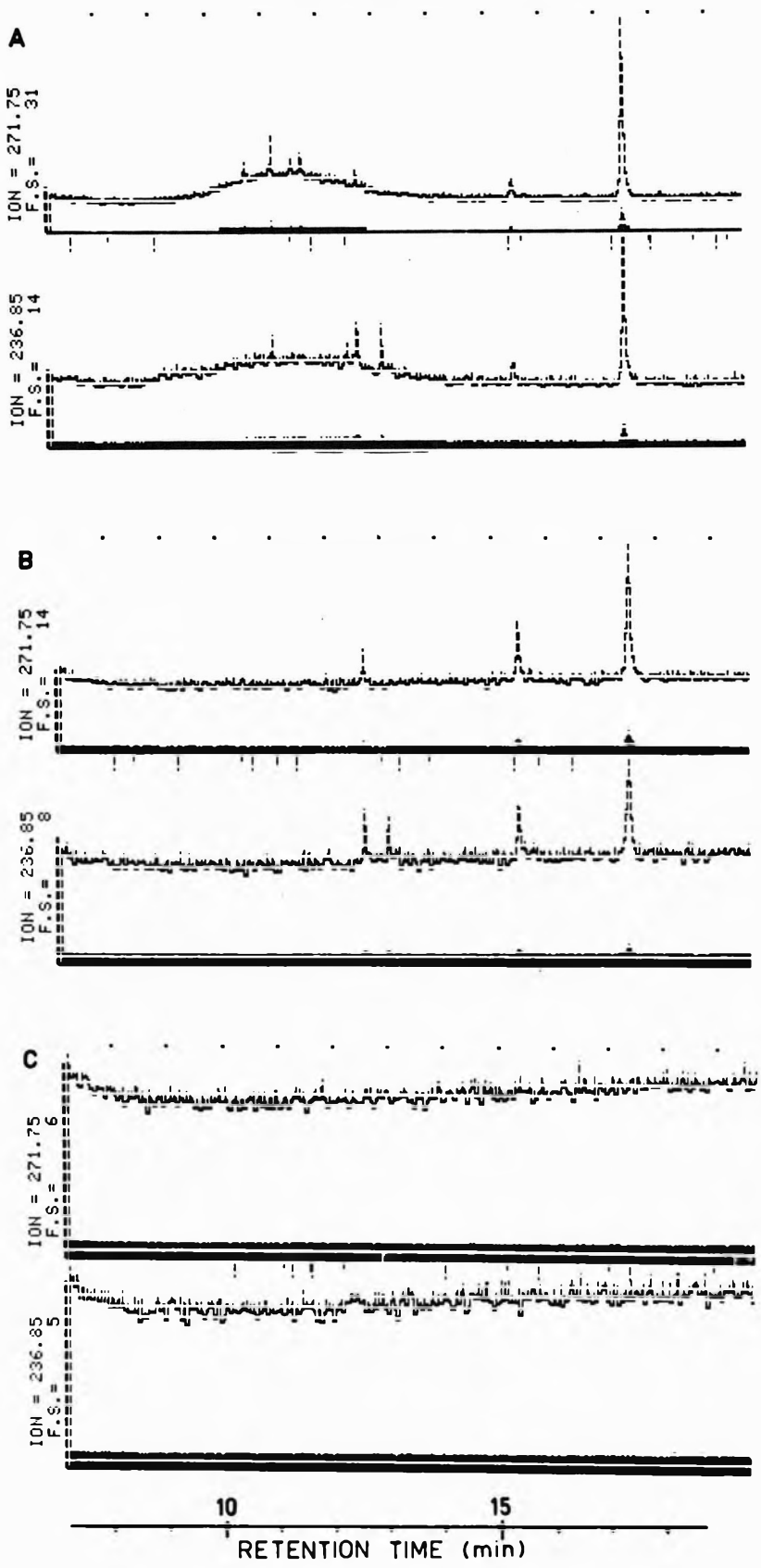

Figure 3. GC-MS-SIM chromatogram for photomirex and mirex of (A) adipose fat extract containing photomirex and mirex equivalent to ca 30 and 100 ng/g, respectively, on fat basis; (B) 1 ng injected of photomirex and mirex; (C) method blank.

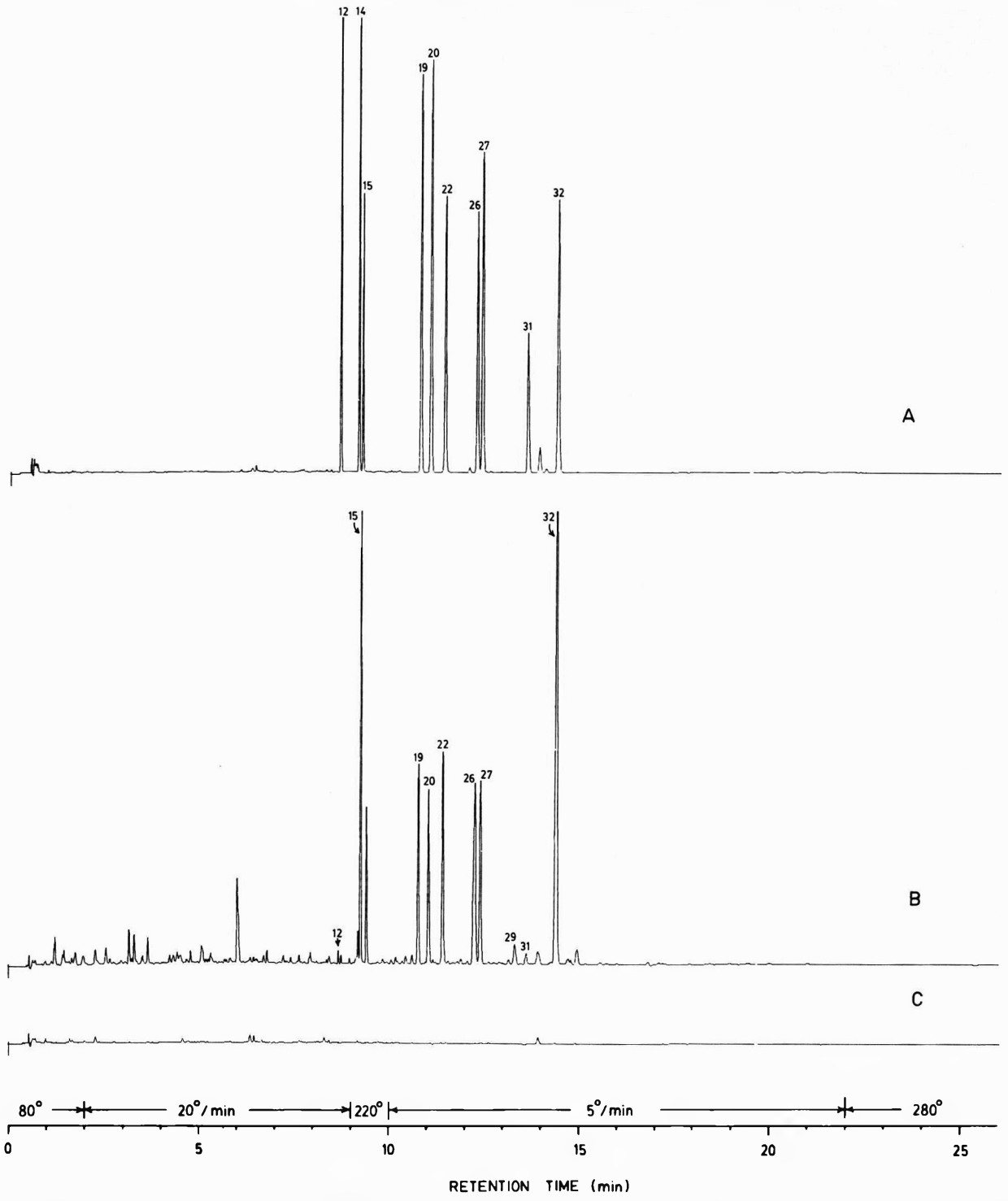

Figure 4. Electron capture GC chromatogram on DB-17 capillary column of (A) OC mixture solution at 50 pg/μL; (B) Florisil Fraction B extract of Kingston composite sample; (C) Florisil Fraction B of method blank. (See Table 1 for peak identification.)

solvent on rotary evaporator in 35°C bath. Weigh extracted fat and transfer to appropriate size glass-stopper graduated cylinder with methylene chloride–cyclohexane (1 + 1, v/v) solvent to obtain 0.2 g fat/mL solution. Mix thoroughly and transfer 1 mL aliquot to preweighed 0.5 dram vial, evaporate solvent with stream of nitrogen, and confirm that extracted fat concentration is 0.2 g/mL. Unless analyzed immediately, seal 6 mL aliquots of fat extracts (1.2 g fat) in 10 mL clean ampules and store at −20°C until analysis.

Gel Permeation Chromatography (GPC)

(a) *Preparation of column.*—Slurry 60 g Bio-Beads S-X3 gel in methylene chloride–cyclohexane (1 + 1, v/v), let swell, and pack GPC column. Adjust bed height to 48 cm.

(b) *Calibration of GPC column.*—As previously described (2).

(c) *GPC fractionation of fat solution.*—Load fat extracts (or fortified extracts) as described in GPC chromatograph manual, with slight modification to minimize waste of scarce fat extract. Load loop with 5.8 mL extract, just enough to completely load sample loop and index to next loop. Rinse lines with GPC solvent before loading with next extract. Collect fraction according to GPC elution volume calibration; typically, discard first 170 mL (34 min) and collect next 130 mL (26 min) containing OC contaminants. Evaporate OC fraction to ca 1–2 mL on rotary evaporator, 35°C water bath, transfer to calibrated centrifuge tube with hexane, and con-

Table 2. Recoveries (% ± SD, duplicate determinations) of organochlorine contaminants

Organochlorine	Fortification level (lipid basis) 10 ng/g	100 ng/g	500 ng/g
1,3,5-Trichloro Bz	63.1± 1.2	64.2± 3.9	57.7±1.6
HCBD[a]	43.3± 1.6	60.1±10.7	62.1±5.1
1,2,3-Trichloro Bz	62.1± 1.7	68.9± 2.9	65.7±0.8
2,4,5-Trichlorotoluene	94.5± 4.6	79.9± 3.2	76.0±0.7
1,2,3,4-Tetrachloro Bz	82.6± 1.4	84.4± 0.5	83.9±1.9
Pentachloro Bz	110.5± 2.9	91.2± 1.6	90.5±1.2
Hexachloro Bz	102.3± 3.1	87.3± 1.8	92.5±0.4
α-BHC	111.5± 4.9	89.2± 3.7	90.5±2.4
Chlordene[a]	75.2± 4.7	85.8± 5.0	93.9±3.0
γ-BHC	88.4± 2.9	96.2± 7.8	125.6±0.4
β-BHC	120.1± 2.1	91.6±11.2	92.7±4.6
Heptachlor[a]	72.4± 4.7	83.7± 7.2	86.9±7.4
Aldrin	97.5± 1.8	85.6± 9.0	96.6±4.1
Octachlorostyrene	102.1± 5.9	91.1± 8.6	93.4±2.9
Oxychlordane[b]	—	81.6± 6.8	96.6±8.3
Heptachlor epoxide[b]	—	85.9± 5.1	98.4±8.4
γ-Chlordane	99.8± 2.8	87.0±10.1	91.5±0.0
Trans-nonachlor[a]	96.6±10.3	90.8± 6.2	91.8±2.1
α-Chlordane	64.0± 2.1	83.9±10.1	90.9±0.5
α-Endosulfan	96.5± 3.3	84.2± 7.6	88.0±0.7
o,*p*'-DDE[a]	109.6± 3.9	90.8± 7.4	89.5±2.4
p,*p*'-DDE	78.9± 2.3	91.1± 9.9	91.8±1.6
Dieldrin	87.7±11.6	93.2±13.2	94.9±3.1
Endrin[a]	126.6±29.1	108.4±11.3	106.0±5.2
Cis-nonachlor[a]	85.3± 7.4	93.8± 9.5	95.9±1.9
p,*p*'-DDD[a]	92.4±18.6	91.2±10.0	93.7±3.1
p,*p*'-DDT	120.9± 5.2	86.0± 7.7	91.6±8.1
Photomirex	106.0± 4.0	93.9±11.7	99.1±0.1
Mirex	106.3± 5.7	96.1± 8.7	98.0±1.1
Methoxychlor[a]	97.5± 9.3	72.3±10.6	70.5±7.0
Decachlorobiphenyl	106.0± 4.5	93.8± 9.7	96.6±3.5
Hexabromobiphenyl	106.0± 4.4	99.7±10.3	95.9±2.0
Aroclor 1260[c]	106.1± 4.4	101.6± 8.6	110.9±6.3

[a]Triplicate determinations.
[b]Four determinations; fortification levels 50 ng/g and 250 ng/g.
[c]Four determinations; fortification levels 250 ng/g and 2500 ng/g.

centrate to 0.3 mL by using gentle stream of nitrogen for further Florisil column cleanup.

Florisil Column Cleanup

Proceed as previously described (2) for separation of OC contaminants to obtain 2 fractions (Fraction A and Fraction B). Concentrate and adjust volume of each fraction to 2 mL in hexane for GC analysis. (Note: Due to wide variations of adsorption activity and density of Florisil, predetermine OC elution volumes for Florisil cleanup by calibration using appropriate OC compounds (2).

Recovery Studies

Obtain clean fat for fortification purposes by collecting first GPC fraction, i.e., ca 50–150 mL fraction, and concentrating to dryness. Fortify GPC-cleaned fat with known amount of standard to give fortification levels in the range 10 to 500 ng/g, on a fat basis. Proceed as described for extraction of adipose fat, GPC cleanup, and Florisil cleanup.

Analysis by GC

Load 0.2 mL aliquot of extract (or standard) solutions into 0.3 mL autosampler microvials and analyze by GC on DB-17 column. For p,p'-DDE analysis, dilute 0.1 mL aliquot to 3 mL to stay within linear range of electron capture detector, then analyze on DB-17 column. Analyze similar aliquots on DB-5 column. Program routines can be used to store chromatographic data on floppy disks for later data processing. Determine amount of unknown or spiked material by comparison of its peak area with corresponding peak of standards.

Confirmation by GC-MS

For selected samples, concentrate portion of 2 mL Florisil column eluate remaining after GC analysis to 0.1 mL, and analyze 2 μL aliquots by GC-MS. Confirm compound identification by monitoring 2 ions per compound, using selected ion monitoring programs. For confirmation of hexabromobiphenyl, inject 6–8 μL aliquots and monitor 4 ions. In all analyses, compare peak heights, on selected ion chromatograms, for each compound in sample with those obtained from standard solution analyzed under similar conditions.

Results and Discussion

The use of GPC with methylene chloride–cyclohexane (1 + 19, v/v) as solvent, although satisfactory for triaryl/alkyl phosphates (2), gave a very wide elution band for OC contaminants, ranging from 80 mL for mirex to 455 mL for β-BHC. However, by using GPC with methylene chloride–cyclohexane (1 + 1, v/v) as solvent, individual OC contaminants could be separated from adipose fat to produce extracts clean enough for direct GC analysis with capillary column and electron capture detection. The GPC elution volumes of OC contaminants are listed in Table 1 and an elution profile of human adipose fat is shown in Figure 1. The OC eluate collection was started as close as possible to the earliest eluting compounds to effect maximum fat isolation. Under the indicated conditions, only about 0.2 mg fat remained in the extract, for 99.98% cleanup efficiency. To facilitate identification and quantitation of the OC contaminants, extracts were further fractionated by Florisil column chromatography (2). Due to the wide variation in density and adsorptivity of the Florisil adsorbent, each batch was calibrated before use (2). The Florisil eluate fraction (A or B) in which the OC compounds occur is indicated in Table 1. The dividing point between Fractions A and B was selected so that *p*,*p*'-DDE was in Fraction A and *p*,*p*'-DDT was in Fraction B. Using

Table 3. Organochlorine contaminant concentration (ng/g, mean ± SD) in human adipose tissue

	Kingston		Ottawa	
Organochlorine	Composite[a] residual	Mean[b] individual	Composite[a] residual	Mean[c] individual
Pentachloro Bz	4.5 ± 3.1	1 ± 2	2.5 ± 0.4	1 ± 5
Hexachloro Bz	156.5 ± 4.3	106 ± 70	82.4 ± 11.4	78 ± 52
α-BHC	3.2 ± 0.2	ND	4.2 ± 0.5	ND
γ-BHC	ND	ND	7.0 ± 1.0	ND
β-BHC	232.8 ± 10.6	136 ± 474	65.7 ± 6.1	65 ± 85
Oxychlordane	59.2 ± 1.6	42 ± 18	47.0 ± 4.5	39 ± 16
Heptachlor epoxide	47.9 ± 1.6	35 ± 20	33.4 ± 3.1	37 ± 21
Trans-nonachlor	115.6 ± 5.0	ND	86.1 ± 8.7	ND
α-Chlordane	ND	18 ± 16	ND	16 ± 6
p,p'-DDE	5547 ± 335	3256 ± 2856	3783 ± 242	2557 ± 2013
Dieldrin	67.2 ± 3.2	36 ± 28	54.2 ± 5.6	43 ± 28
Cis-nonachlor	13.9 ± 0.7	ND	9.5 ± 1.3	ND
p,p'-DDD (+ *o,p'*-DDT)	12.2 ± 1.5	14 ± 11	26.6 ± 3.2	9 ± 9
p,p'-DDT	126.0 ± 11.2	159 ± 156	157.5 ± 12.7	128 ± 107
Photomirex	7.0 ± 0.6[d]	9 ± 11	ND[d]	6 ± 4
Mirex	37.3 ± 5.5	27 ± 38	16.6 ± 2.4	11 ± 16
Aroclor 1260	2608 ± 73	2950 ± 3626	1814 ± 217	2001 ± 873

[a]Four determinations of composite sample.
[b]Single determination on 91 samples.
[c]Single determination on 84 samples.
[d]GC-MS-SIM analysis.

this scheme, only *trans*-nonachlor, *o,p'*-DDE, and *o,p'*-DDT were split between the 2 fractions.

Fractions A and B were separately analyzed by capillary GC on a DB-17 (50% methyl/phenyl silicone) column. This column was chosen as the primary column because most OC compounds could be resolved using a relatively short 15 m column. Only mirex and photomirex were not adequately resolved from the polychlorinated biphenyl peaks and *p,p'*-DDD and *o,p'*-DDT had the same retention time. However, mirex could be resolved from PCB and other OC contaminants by using the relatively nonpolar DB-5 column, although on this column other OC contaminants had overlapping peaks (i.e., oxychlordane/heptachlor epoxide; *cis*-nonachlor/*o,p'*-DDT). A DB-1 column did not provide better separation than the DB-5 column. The DB-17 column also allowed detection of *cis*-nonachlor which co-eluted with *p,p'*-TDE/*o,p'*-DDT on the less-polar columns. Retention times for the OC contaminants on the DB-17 and DB-5 columns are listed in Table 1 and typical chromatograms are illustrated in Figures 2A and 2B.

The identity of the contaminants was confirmed on a DB-17 column by GC-MS selected ion monitoring using 2 ions per compound (Table 1). Because of the low levels of OC contaminants, the MS confirmation was usually only semiquantitative and reported concentrations are based on electron capture GC quantitation, except for photomirex, which could not be detected because of interference from other compounds. Typical selected ion chromatograms are shown for mirex and photomirex for a sample (Figure 3A), standards (Figure 3B), and a sample blank (Figure 3C). Selected ion chromatograms for ions of other compounds were similar to these.

Recovery studies were carried out on GPC-cleaned fat fortified before the extraction stage with OC mixtures to obtain, for each OC compound, fortification levels in the range 10 to 500 ng/g on extracted fat basis. The recoveries were all essentially quantitative (≥ 80%) except for hexachlorobutadiene and the more volatile trichlorobenzenes (60%). Recoveries of Aroclor 1260 from extracts fortified at 250 and 2500 ng/g were also essentially quantitative. The percentage recoveries of the PCB and OC compounds in the fortification studies are listed in Table 2.

To evaluate the method, 4 replicates of each of 2 composite human fat extracts were analyzed for all the contaminants investigated. The extracts were analyzed by electron capture GC on both a DB-17 and a DB-5 fused silica capillary column and confirmed by GC-MS selected ion monitoring. The results of the analyses are reported in Table 3 and indicate a relative standard deviation of better than ± 10% for almost all compounds. Typical DB-17 chromatograms of the Florisil Fractions A and B extracts are illustrated in Figures 2C and 4B, respectively, together with appropriate standards (Figures 2A, 2B, 4A) and blanks (Figures 2D, 4C). Typical GC-MS selected ion chromatograms used for confirmation of the identity are shown for mirex and photomirex (Figures 3A, 3B, 3C). The composite samples were obtained by combining residual tissue samples from a previous study of human adipose tissue (1). Since equal amounts of each individual tissue were not combined, the contaminant levels in the composite samples will not be exactly the same as the mean values reported for the individual tissues. However, the contaminants identified should be the same and their concentrations should be of the same magnitude. Table 3 lists those contaminants identified in the previous survey and their mean concentrations. The results of the 2 sets of analyses are consistent except for *trans*-nonachlor which had been misidentified as α-chlordane in the earlier survey (1) because of similarity of retention times and a nonselective ion (m/z 35) chosen for MS confirmation. The superior resolution of the DB-17 column clearly separated the 2 compounds and selected ion monitoring with appropriate ions (Table 1) easily distinguished the 2 compounds in this present study. The detection of *trans*-nonachlor in both Fractions A and B also confirms its identity.

The *cis*-nonachlor isomer was also detected in the tissue extract by electron capture GC on the DB-17 column. This isomer has not usually been reported in other surveys probably because it co-elutes with *o,p'*-DDT on the nonpolar columns used in earlier studies, although Wright et al. (9) have previously reported the presence of a "*cis*-nonachlor" in human adipose tissue.

In conclusion a semiautomated GPC method combined with dual capillary column GC has been developed for the rapid analysis of OC contaminants in human adipose tissue.

The method illustrates the usefulness of semiautomation to improve the speed and reproducibility of analytical methods.

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Determination of Glyphosate Herbicide and (Aminomethyl)phosphonic Acid in Natural Waters by Liquid Chromatography Using Pre-Column Fluorogenic Labeling with 9-Fluorenylmethyl Chloroformate

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An analytical method has been developed for determination of glyphosate herbicide and its major metabolite, (aminomethyl)phosphonic acid (AMPA), in natural waters. Sample pretreatment consisted of filtration, addition of phosphate buffer, concentration by rotary evaporation, and a final filtration before derivatization with 9-fluorenylmethyl chloroformate. The derivatives were separated by anion exchange liquid chromatography and measured with a fluorescence detector. Standard curves were linear over 3 orders of magnitude and minimal detectable quantities were 10 ng/mL for glyphosate and 5 ng/mL for AMPA. The 20-fold concentration factor realized in sample preparation corresponds to ppb method detection limits for glyphosate and AMPA in natural waters. Recovery and storage studies were performed and are discussed.

Glyphosate [*N*-(phosphonomethyl) glycine; Roundup®] is a broad spectrum, nonselective, post-emergence herbicide that has found widespread agricultural and domestic use. Recently, it has been introduced for the control of aquatic weeds (Rodeo®). Analytical methods development for the determination of glyphosate in environmental samples has not been avidly pursued, largely because of its low mammalian toxicity (LD_{50} = 1568 mg/kg) and subsequent low risk of environmental pollution. Nevertheless, the effect of glyphosate on nontarget organisms and its overall environmental fate cannot be fully evaluated unless techniques possessing suitable sensitivity and selectivity are available.

Several chromatographic methods have been developed for the analysis of glyphosate and its major metabolite, (aminomethyl)phosphonic acid (AMPA), including gas chromatography (GC) after chemical derivatization (1–3), thin layer chromatography (4, 5), and liquid chromatography (LC; 6, 7). Recently, these methods and several others have been reviewed (8). The ionic, water-soluble character of glyphosate and AMPA make analysis by LC advantageous over GC. Although glyphosate and AMPA cannot be sensitively measured by conventional photometric LC detectors, highly fluorescent derivatives can be formed pre-column, using 9-fluorenylmethyl chloroformate (FMOCC1) (6), or post-column with orthophthalaldehyde-mercaptoethanol (OPA-MERC) (7). The post-column procedure forms derivatives on-line but it requires more instrumentation and careful maintenance. Conversely, the pre-column method is rapid and simple and requires minimal equipment and analyst experience.

Analysis of glyphosate and AMPA by LC as FMOC derivatives has been applied to vegetation (9, 10) and water and soil (11). Glass (11) reported good recoveries and detection limits for glyphosate in water but the procedure required ion-exchange cleanup and AMPA was not determined. We have applied this pre-column LC procedure to the analysis of glyphosate residues in natural waters. We report a shortened sample preparation and include the determination of AMPA. Method limitations and recoveries from fortified water samples are discussed.

Experimental

Sample Preparation

Natural waters investigated included rainwater, lake water, and river water from a forest watershed; samples were collected and frozen in polyethylene bottles until analyzed. Periodically, frozen samples were thawed and thoroughly shaken to mix, and about 150 mL was filtered through Whatman No. 1 paper. In recovery experiments, samples were fortified with herbicide and metabolite before this filtration. A 100 mL aliquot of this water was placed into 250 mL round-bottom flask and 1 mL 0.1M K_2HPO_4 was added. Samples were concentrated to near-dryness by rotary evaporation (Buchi Model R; Brinkmann) at 30–50°C and diluted to 5.0 mL by carefully rinsing the flask twice with 2 mL washes of deion-

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Table 1. Capacity factors for AMPA and glyphosate derivatives on selected columns[a]

Column[b]	Derivative	
	AMPA	Glyphosate
μNH_2 (Alltech)	1.14	2.45
$N(CH_3)_2$ (Macherey-Nagel)	1.38	1.77
μCarbohydrate (Waters)[c]	1.10	3.10
SAX (Altex)	0.70	>5.7

[a]75% v/v mixture of 0.05M KH_2PO_4 (pH 6.0) in acetonitrile.
[b]SAX column is a strong anion exchanger; all others are weak anion exchangers.
[c]75% v/v mixture of 0.1M KH_2PO_4 (pH 6.0) in acetonitrile.

ized water. They were stored at 4°C if not analyzed immediately.

Standard Preparation

Fifty mg glyphosate (Monsanto) or AMPA (Sigma) was dissolved in 500 mL deionized water to yield 100 μg/mL stock solutions. Mixed standards (glyphosate and AMPA) covering the range of 0.005–10 μg/mL were prepared by appropriate dilutions of the stock solutions in deionized water. Standard solutions were refrigerated and no degradation was observed over 6 months.

Derivatization

Approximately 0.5–1 mL sample concentrate was passed through a Gelman GA-8 (0.2 μm) filter and into a 2 mL glass vial. A 0.10 mL aliquot of filtered sample was placed in a small glass culture tube, followed by 0.90 mL 0.025M borate buffer, 0.90 mL LC grade acetone, and 0.10 mL 0.01M 9-fluorenylmethyl chloroformate (FMOCC1; Aldrich) in acetone. Tubes were shaken and allowed to react 20 min at room temperature. Excess reagent was removed by three 1 mL washes of ethyl ether (top layer). Samples were analyzed within 8 h.

Liquid Chromatography

The instrument used consisted of an Altex Model 110A pump, Rheodyne Model 7125 injector (200 μL loop), 0.4 × 25 cm μNH_2 column (Alltech Assoc.), Aminco spectrophotofluorometer (excitation 270 nm; emission 315 nm) equipped with 50 μL flow cell, and Soltec strip chart recorder (50 mV). Mobile phase consisted of 75% (v/v) mixture of 0.05M KH_2PO_4 (pH 6.0 with KOH) in acetonitrile (Fisher LC grade) delivered at 1.0 mL/min. Glyphosate and AMPA were measured by comparing peak heights of samples to external standard curve of at least 3 points.

During the development of this method, the following LC columns (all 0.4 × 25 cm) were also evaluated: $N(CH_3)_2$ (Macherey-Nagel), μCarbohydrate (Waters), and SAX (Altex). Also, a Gilson Specta/Glo filter fluorometer (excitation 280 nm; emission 300–400 nm) equipped with 8 μL flow cell was compared with the spectrophotofluorometric instrument.

Storage Study

Two separate storage studies were performed. Control water samples with added propionic acid (3 mL/300 mL sample) were fortified with glyphosate and AMPA at 0.05 and 0.50 ppm and stored in a refrigerator (4°C) for 3 months. Control water samples (no propionic acid) were fortified with glyphosate at 0.05 and 0.50 ppm and frozen (0°C) for 3 months. Subsequently, these samples were prepared and analyzed as described above.

Results and Discussion

Since glyphosate and AMPA are zwitterionic molecules, formation of their respective FMOC derivatives by reaction of the amine (analyte) and the acid chloride (FMOCC1) yields anionic compounds which can easily be separated by anion exchange liquid chromatography (6). Several anion exchange stationary phases were examined, and in all cases, AMPA-FMOC, the weaker acid, eluted before glyphosate-FMOC (Table 1). Efficiency on all of these columns ranged from 2000 to 3000 theoretical plates. For the 4 different stationary phases examined, maximum retention of AMPA-FMOC was observed with the dimethylamine moiety ($N(CH_3)_2$), while glyphosate-FMOC was retained longest on the SAX column under the conditions tested. These conditions (0.1–0.05M phosphate buffer with 25% acetonitrile) offered the best compromise between good sensitivity and reasonable retention time. Phosphate buffer was the only salt evaluated and other buffers could significantly affect separation behavior. Substitution of methanol for acetonitrile resulted in significant deterioration of efficiency.

Practical application of chromatographic methods to environmental samples requires that analyte retention be controlled such that interference peaks can be circumvented. In anion exchange, retention usually can be increased by a decrease in ionic strength of buffer and/or an increase in pH (12). On silica-based stationary phases, a decrease in the percentage of organic modifier will also increase retention. Our experience with the columns examined has been that a decrease in ionic strength or percentage of acetonitrile increases retention at the expense of significantly degraded peak shape and sensitivity (see Figure 1).

Glyphosate-FMOC retention could easily be controlled by changing the buffer pH, especially on the SAX column. However, for AMPA-FMOC, varying the pH did not significantly increase retention on any of the stationary phases evaluated. Retention increased slightly from pH 4 to 6, but decreased as pH was increased to 8. Roseboom and Berkhoff (10) reported that the mobile phase pH (5–8) had no effect on the retention

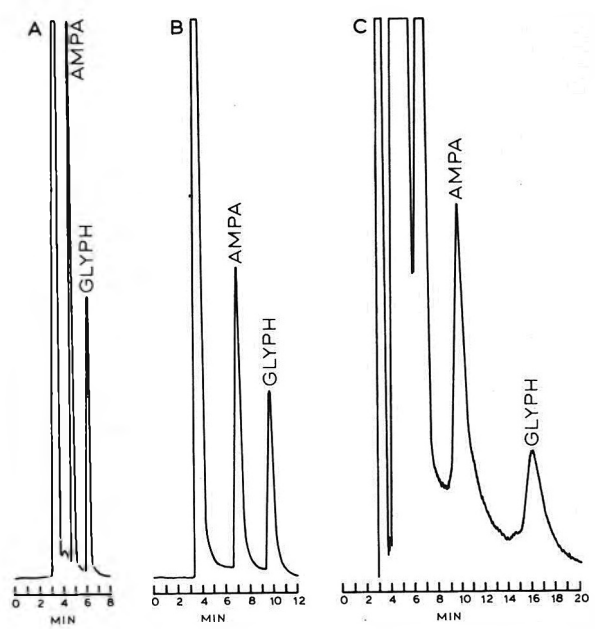

Figure 1. Chromatograms of AMPA-FMOC and glyphosate-FMOC standards on μNH_2 column with different mobile phases, demonstrating decrease in efficiency with decreased percent organic modifier or buffer ionic strength: (A) 75% 0.10M KH_2PO_4, pH 6/25% CH_3CN; (B) 90% 0.10M KH_2PO_4, pH 6/10% CH_3CN; (C) 90% 0.05M KH_2PO_4 pH 6/10% CH_3CN.

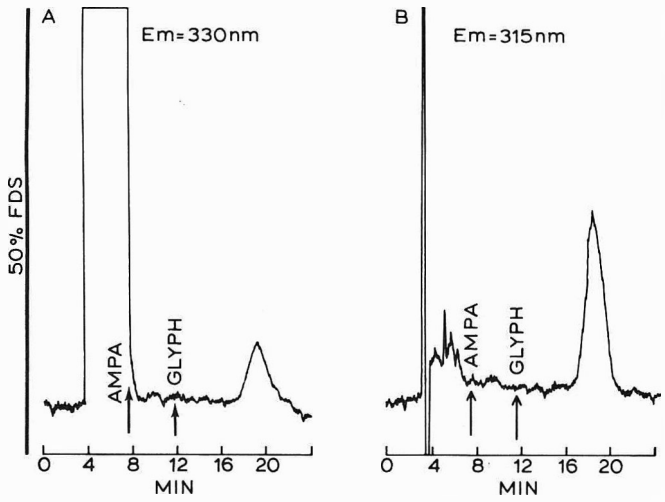

Figure 2. Chromatograms of derivatized control forest water showing effect of emission wavelength on interference peaks (excitation wavelength 295 nm; mobile phase 75% 0.05M KH_2PO_4, pH 6/25% CH_3CN; μNH_2 column).

of AMPA-FMOC on a Hypersil APS column. On the weak anion exchange columns, the cationic character of the stationary phase decreases as pH approaches the pK value (about 9); thus retention should decrease as pH is increased above 6. On the strong anion exchange column (SAX), the cationic character should not significantly decrease until the pH is greater than about 9, but increasing the mobile phase pH did not increase retention of AMPA-FMOC on this column. This was unfortunate because AMPA-FMOC eluted very early in the chromatogram which increased the possibility for potential interferences. This is further confounded by the fact that AMPA resembles many amino acids which are certain to be found in most agricultural samples. For better control of AMPA-FMOC retention by anion exchange, a change in the buffer salt offers a good possibility. Since multi-charged ions are generally held on ion exchangers more strongly, phosphate buffers at a high pH will compete strongly for ion exchange sites on the stationary phase. It is also possible to separate the AMPA-FMOC and glyphosate-FMOC derivatives by ion-pair or micellar liquid chromatography.

Two fluorescence detectors were evaluated; a filter fluorometer and a spectrophotofluorometer (SPF). Typically, filter fluorometers offer better sensitivity because of a higher optical transmissivity while the SPF has better selectivity because of the narrow bandpass. Nevertheless, for the 2 systems evaluated here, the spectrophotofluorometric instrument was 20–50 times more sensitive. One reason for this difference was its larger diameter light path (50 μL cell) compared to the filter fluorometer (8 μL). A smaller cell decreases band spreading in an LC detector, but the relatively wide peaks that are typical of ion-exchange LC are usually not significantly affected by larger cells. Another important difference in these 2 detectors was the higher intensity of the spectrophotofluorometric light source (200 W xenon arc) compared to the filter fluorometer (5 W mercury lamp). In addition, it was found that the selectivity of the spectrophotofluorometer allowed spectral resolution of some sample interferences. A shift in emission wavelength from 330 to 315 nm virtually eliminated forest water sample interferences that eluted early from the column (Figure 2). It should also be noted that these derivatives are good chromophores and can be detected by UV absorbance at 263 nm, however, with a significant sacrifice in sensitivity.

For most of our applications, we chose the μNH_2 (Alltech) column because it gave good separation and the cost was about ⅓ that of the other columns tested. With this column, a mobile phase of 0.05M phosphate (pH 6.0), and the spectrophotofluorometric detector, standard curves for glyphosate and AMPA were linear from about 0.01 to 10 μg/mL, or 3 orders of magnitude. Minimum detectable quantities (S/N = 3) were about 0.01 μg/mL (0.1 ng) for glyphosate = FMOC and 0.005 μg/mL (0.05 ng) for AMPA-FMOC (example calculation: 0.1 mL of a 0.01 μg/mL glyphosate standard is 0.001 μg; 0.001 μg in a total of 2 mL derivatizing solution yields a 0.0005 μg/mL glyphosate-FMOC solution; a 0.2 mL injection of that solution is 0.0001 μg or 0.1 ng). The 20-fold concentration factor achieved in sample preparation allows ppb method detection limits in natural water samples.

For analysis of the natural waters examined, sample preparation was minimal. Sample preparation by filtration, rotary evaporation, and a final filtration took about 1.5 h/sample and achieved a 20-fold concentration factor. In contrast, the ion exchange cleanup used previously (11) would take much longer to realize a similar concentration factor in addition to the expense and preparation time of the ion exchange resin. Furthermore, Glass (11) did not determine if AMPA was quantitatively recovered by the ion exchange method.

During the evaluation of our preparation procedure, recoveries of glyphosate from fortified deionized water were inconsistent. Since glyphosate is known to adsorb strongly to soils (5, 13), we believe that similar adsorption to glass surfaces was responsible for irregular recoveries. Subsequently, we found that addition of phosphate buffer to the water sample before concentration resulted in higher and more reproducible recoveries (see Table 2). It appears that inorganic phosphate competes with glyphosate for binding sites on glass, thereby minimizing adsorption of the analyte.

All determinations were considered to be "free" glyphosate and AMPA since filtration would remove the sorbed fraction. The waters reported here had small amounts of particulates and recoveries were good, suggesting that sorption was minimal. Waters with high levels of suspended matter, especially clays, would probably lose significant amounts of glyphosate and AMPA through filtration.

Also, the temperature of the sample may have been affecting recoveries during the rotary evaporation step. However, concentration of duplicate solutions fortified with glyphosate (plus phosphate) at 30, 40, and 50°C showed no significant differences in recovery, indicating that temperature over this range is not critical to good recovery. It should be noted that the rate of concentration was fastest at 50°C; this temperature was used throughout the course of this study.

Recovery of glyphosate and AMPA from fortified natural waters was good at all levels tested (Table 2). Standard devia-

Table 2. Recovery of AMPA and glyphosate from fortified forest water samples, using phosphate buffer addition before concentration

Level of spike, ppm		Av. rec., % AMPA	Av. rec., % Glyphosate
0.010	(n = 3)	NA[a]	111 (RSD 6%)
0.050	(n = 8)	80 (RSD 15%)	76 (RSD 16%)
0.50	(n = 3 AMPA) (n = 6 GLYPH)	100 (RSD 24%)	91 (RSD 7%)
5.0	(n = 3)	97 (RSD 7%)	96 (RSD 10%)

[a]NA = Not analyzed in triplicate.

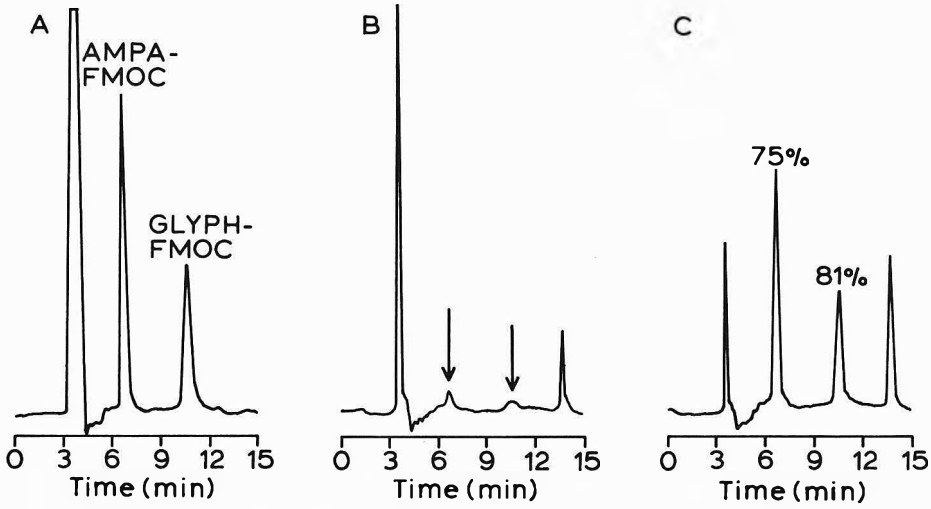

Figure 3. Chromatograms of (A) 25 ng each AMPA-FMOC and glyphosate-FMOC, (B) forest water sample (ca 2 ppb glyphosate and AMPA), and (C) same forest water fortified with 50 ppb glyphosate and AMPA (percent recoveries are listed above peaks).

tions were acceptable with the exception of AMPA at the 0.50 ppm level. In many water samples analyzed, interferences eluted at or near the AMPA-FMOC retention time, which made quantitation difficult, especially at lower AMPA concentrations. No interferences were observed for glyphosate-FMOC in the samples analyzed, but many samples had a peak that eluted after glyphosate-FMOC (ca 15 min; see Figure 3).

Propionic acid can act as a bactericide and we evaluated its action as a preservative for glyphosate and AMPA in water. Fortified samples that were treated with propionic acid and stored in a refrigerator (4°C) for 3 months showed significant loss of glyphosate and AMPA. For triplicate natural water samples fortified at 0.05 ppm, recoveries averaged 45% (RSD 29%) for glyphosate and 109% (RSD 32%) for AMPA. These results suggest that glyphosate was degraded to AMPA. For triplicate natural water samples fortified at 0.50 ppm, recoveries of glyphosate averaged 73% (RSD 20%) while AMPA recoveries averaged 30% (RSD 68%). The results of this storage study indicate that addition of propionic acid and refrigeration of water samples at 4°C is not sufficient to retard degradation of glyphosate for long periods.

Sometimes sample concentrates could not be analyzed immediately and were refrigerated for up to 2 weeks. To ensure stability of glyphosate, spiked samples were reanalyzed periodically. No significant decrease was observed in spiked water sample concentrates stored in the refrigerator for up to one month.

Subsequent experiments where glyphosate was fortified into a natural water (no propionic acid) and frozen at 0°C for 3 months showed that it was not significantly degraded. For duplicate natural water samples fortified at 0.05 and 0.50 ppm, recoveries of glyphosate averaged 81% (RSD 11%) and 86% (RSD 3%), respectively. Thus, freezing water samples as soon as possible after collection is suggested to ensure the stability of glyphosate in samples to be analyzed at a later date.

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Rapid, Semimicro Method for Determination of Polycyclic Aromatic Hydrocarbons in Shellfish by Automated Gel Permeation/Liquid Chromatography

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A simple, rapid, easily automated method is described for the determination of polycyclic aromatic hydrocarbons (PAHs) in shellfish such as American lobster *(Homarus americanus)* and blue mussel *(Mytilus edulis)*. PAHs are extracted from small amounts (1–8 g) of tissue by saponification in 1N ethanolic potassium hydroxide followed by partitioning into 2,2,4-trimethylpentane. This solution is evaporated just to dryness by rotary evaporation and the residue is dissolved in cyclohexane–dichloromethane (1 + 1) for gel permeation chromatography (GPC) on Bio-Beads SX-3. The GPC procedure is ideal as a screening method in the range 25–18 000 ng PAHs/g tissue. If individual PAH measurements are required, the appropriate GPC fraction is collected and PAHs are separated by reverse phase liquid chromatography (LC) with fluorometric detection. Individual PAHs at concentrations as low as 0.25–10 ng/g can be determined. Recoveries of added fluoranthene, pyrene, benz[*a*]anthracene, chrysene, benzo[*e*]pyrene, benzo[*b*]fluoranthene, benzo[*k*]fluoranthene, benzo[*a*]pyrene, dibenz[*a,h*]anthracene, benzo[*ghi*]perylene, and indeno[1,2,3-*cd*]pyrene were quantitative, with relative standard deviations ranging from 0.0 to 16.9%.

A variety of methods for the determination of polycyclic aromatic hydrocarbons (PAHs) in biological matrices has been reported (1–5). Most reported methods have been developed for the analysis of PAHs at very low (ng/g) levels and, as a consequence, large sample sizes (20–100 g) are used. This in turn dictates the use of large quantities of expensive reagents such as Florisil (2, 5) and high purity solvents. Some of these methods use undesirable solvents such as benzene (2) which is toxic, or toluene (5), the high boiling point of which makes removal by evaporation difficult. Other undesirable features include multiple liquid-liquid partitions (1, 5) which can produce emulsions and low recoveries of some PAHs (4, 5), and lengthy liquid chromatography (LC) procedures (4). Many methods also involve the cleaning of large amounts of relatively complex glassware.

This paper describes a method for the isolation and measurement of PAHs from more highly contaminated marine shellfish (25–18 000 ng PAHs/g) which eliminates the undesirable features mentioned above. These studies also indicate that the method is satisfactory for measuring individual PAHs in the 0.25–10 ng/g range.

METHODS

Apparatus

(a) *Saponification.*—Pyrex Folin-Wu NPN tubes (nonprotein nitrogen tubes or similar 200 mm × 25 mm od digestion tubes), Labconco micro-Kjeldahl digestion rack with flask support rod sized to support Folin-Wu tubes, 100 mL glass-stopper graduated mixing cylinders, disposable Pasteur pipets with long (12 cm) tips, 10 mL glass syringe with ground glass Luer tip, 100 mL ⅀24/40 round-bottom flasks, Büchi all-glass/Teflon rotary evaporator with water bath at 35°C. Rotary evaporator is modified so that the air bleed is replaced with a greaseless Teflon stopcock with Teflon tube extending the length of the steam duct and a glass wool plug at the distal end to filter dust from laboratory air introduced through the bleed.

(b) *Gel permeation chromatograph.*—Autoprep 1001 (ABC Laboratories, Columbia, MO) with 45 cm × 2.5 cm id water-jacketed glass column and solvent-resistant plungers, filled with slurry of 50 g 200–400 mesh Bio-Beads SX-3 in dichloromethane–cyclohexane (1 + 1, v/v) and compressed to bed length of 30 cm. Column was maintained at 25°C with a water bath. Chromatograph was equipped with Schoeffel Model GM 770 variable wavelength monochromator set at 254 nm and Schoeffel Model SF 770 Spectroflow Monitor (Kratos, Inc., Westwood, NJ). A second detector, Waters differential refractometer R403 (Waters Associates Ltd, Milford, MA), was connected in series to the Schoeffel UV detector.

(c) *Small volume make-up device.*—Urinary sediment tube or Shevky-Stafford albumin tube.

(d) *Liquid chromatograph.*—Waters Model 721 system controller and data module; Waters WISP 710B injector; 2 Waters M6000A chromatography pumps; 15 cm × 4.6 mm id column packed with Vydac 201 TP54 reverse phase C_{18} (The Separations Group, Hesperia, CA) maintained at 23.5°C

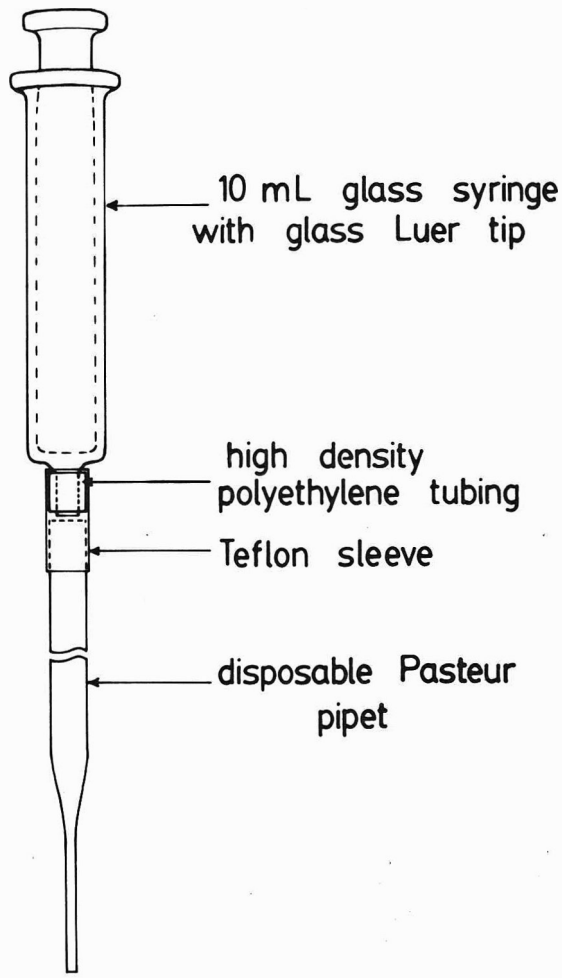

Figure 1. Pipet controller used for transfer of solutions.

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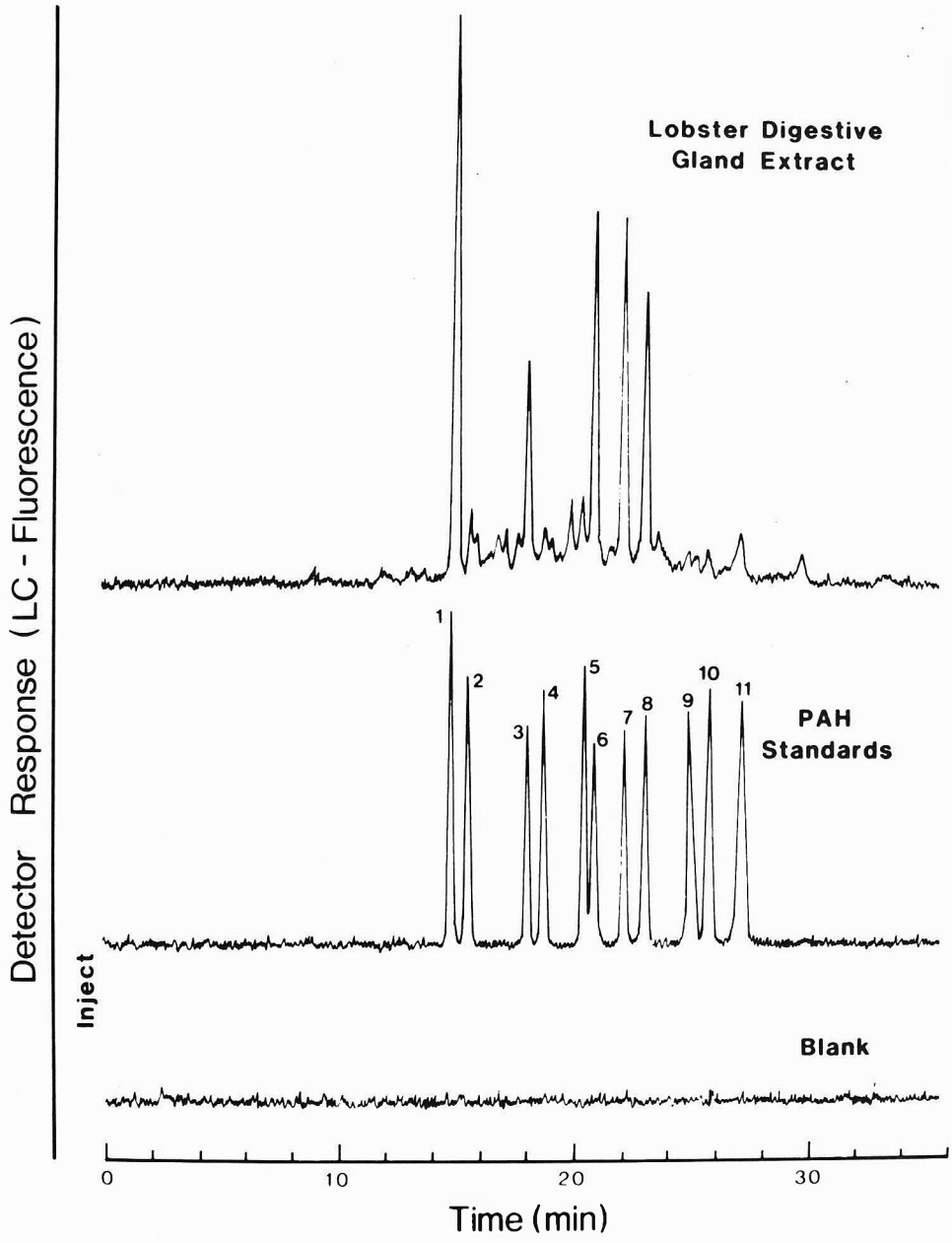

Figure 2. LC chromatogram (fluorescence detector) of standards and typical sample of contaminated lobster digestive gland. PAH standards: (1) 2.4 ng fluoranthene; (2) 13.0 ng pyrene; (3) 1.1 ng benz[a]anthracene; (4) 12.0 ng chrysene; (5) 11.0 ng benzo[e]pyrene; (6) 1.0 ng benzo[*b*]fluoranthene; (7) 0.5 ng benzo[*k*]fluoranthene; (8) 1.3 ng benzo[a]pyrene; (9) 4.0 ng dibenz[*a,h*]anthracene; (10) 7.6 ng benzo[*ghi*]perylene; (11) 5.0 ng indeno [1,2,3-*cd*]pyrene. Injection volume, 10 μL.

by LC-22 column temperature controller (Bioanalytical Systems Inc.); Schoeffel Model FS970 LC fluorometer and Model GM 970 monochromator (λ(ex) = 280 nm, λ(em) = 389 nm); and Kratos Model SF 770A Spectroflow Monitor with GM 770A monochromator set at 254 nm.

Reagents

(a) *Solvents.*—Ethanol, 95% (Consolidated Alcohols, Toronto, Ontario), redistilled in glass (center cut); 2,2,4-trimethylpentane (isooctane), dichloromethane, cyclohexane, all distilled-in-glass grade (Caledon Laboratories, Georgetown, Ontario), acetonitrile, LC grade (Fisher Scientific Co., Fairlawn, NJ); water, methanol, chloroform, all LC grade (Caledon Laboratories).

(b) *Bio-Beads SX-3.*—Bio-Rad Laboratories, Richmond, CA.

(c) *Potassium hydroxide.*—Fisher Certified.

(d) *PAH standards.*—Fluoranthene, benzo[*a*]pyrene, benzo[*b*]fluoranthene, benzo[*k*]fluoranthene, benzo[*ghi*]perylene, indeno[1,2,3-*cd*]pyrene, benz[*a*]anthracene, dibenz [*a,h*]anthracene (Supelco, Inc., Bellefonte, PA). Benzo [*e*]pyrene and pyrene (Analabs, Inc., North Haven, CT). Chrysene (courtesy of John Farrington, Woods Hole Oceanographic Institute). Spiking standard for recovery studies, Supelco Mixture 610-M.

(e) *Boiling chips.*—Anti-bumping granules (BDH Chemicals, Toronto, Ontario).

(f) *Paraffin oil.*—White, light, laboratory grade (Fisher Scientific Co.).

(g) *Nitrogen.*—Laboratory grade, purified by passing through activated charcoal and molecular sieves 5A.

Saponification

For high-fat or highly contaminated tissues such as homogenates of lobster digestive glands or whole mussel soft tissues containing total of ca 100–20 000 ng PAHs/g sample, weigh 1–2 g tissue into bottom of Folin-Wu tube. Add 1.5 g potassium hydroxide, 25 mL redistilled 95% ethanol, and several

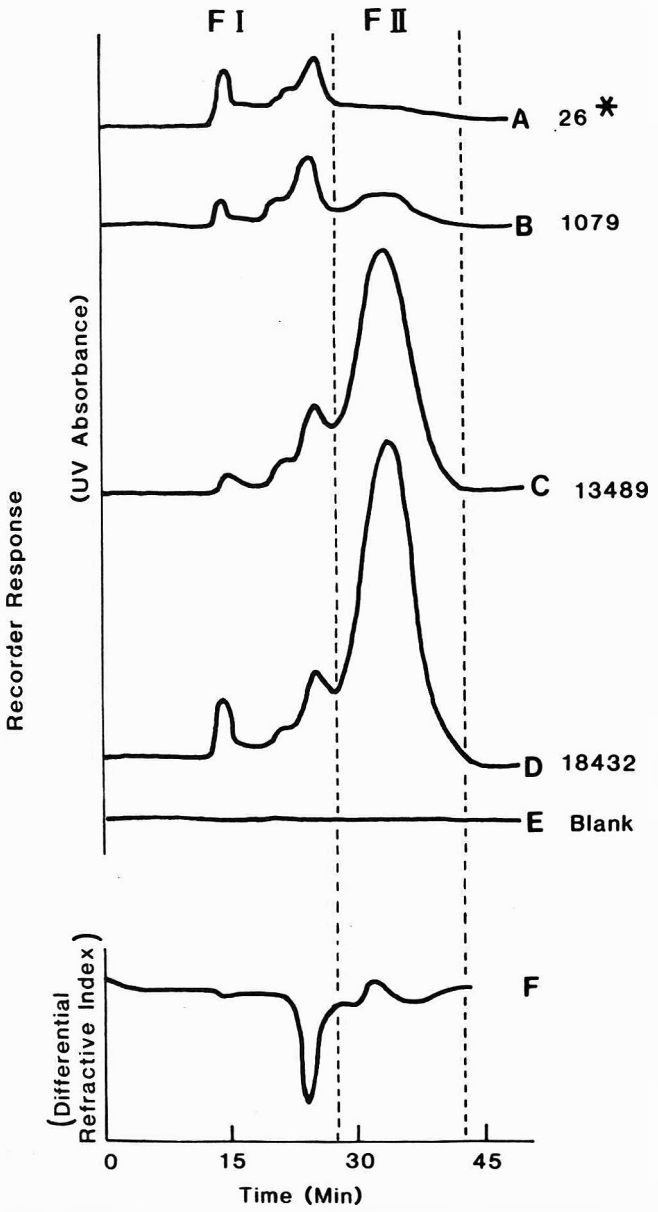

Figure 3. Gel permeation chromatograms (UV detection) of extracts of saponified digestive glands of lobsters taken at various distances from coking plant.

D, closest to plant; C, intermediate distance; B and A, farthest and approximately equidistant from plant; F, GPC-refractive index counterpart of curve D. *Sum of 10 PAHs (listed in Table 2) in ng/g wet weight of tissue.

anti-bumping granules. Reflux gently 2 h on micro-Kjeldahl digestion rack (upper part of Folin-Wu tube acts as reflux condenser). Volume of ethanol should be maintained by adding fresh redistilled ethanol if required, which prevents formation of emulsions during subsequent extraction step. If lowest setting on heater is still too high, resulting in solvent loss during reflux, place small piece of aluminum foil between bottom of tube and heating element to impair heat transfer. For low-fat tissues such as lobster tail or claw muscle with low PAH concentrations, weigh 8 g piece of whole tissue in Folin-Wu tube containing 25 mL 1N freshly prepared ethanolic potassium hydroxide, cover tube with aluminum foil, and leave overnight at room temperature; this results in complete dissolution of tissue. Carry out usual reflux and extraction steps the following morning.

Extraction

While hot, add contents of Folin-Wu tube to 100 mL glass-stopper graduated mixing cylinder containing 25 mL LC grade water and rinse Folin-Wu tube twice with 4 mL redistilled ethanol and once with 10 mL 2,2,4-trimethylpentane, adding rinsings to 100 mL cylinder. Shake stoppered cylinder vigorously, let layers separate, and transfer upper layer to 100 mL round-bottom flask, using a clean disposable Pasteur pipet joined to 10 mL glass syringe by Teflon sleeve (Figure 1) as pipet controller (J. Solomon, Fisheries and Oceans Canada, Winnipeg, Manitoba, Canada, unpublished). Use only disposable pipet to contain the solution (not syringe) and be sure that tip of pipet is cut square so that at least 95% of the upper layer can be transferred. Re-extract lower layer with two 10 mL portions of 2,2,4-trimethylpentane and transfer upper phase to 100 mL round-bottom flask as described above. Rotary-evaporate this solution CAREFULLY just to dryness and add 8.0 mL cyclohexane–dichloromethane (1 + 1, v/v). Ensure that this solvent composition is the same as that of GPC eluting solvent to prevent production of an artifact. (Losses during evaporation procedure will be negligible if water bath is lowered away from flask while a few drops of solvent remain in flask. Simultaneously, slowly open air bleed on distal end of rotary evaporator, modified with Teflon stopcock to allow slow bleed of laboratory air filtered through glass wool. Thus, vacuum inside flask is lowered while flask is cooled by vaporization of residual solvent, and PAH residue is not volatilized.)

Gel Permeation Chromatography

Inject solution into GPC apparatus and elute with dichloromethane–cyclohexane (1 + 1, v/v) pumped at 4.5 mL/min at 8 psig. Maintain column temperature and differential refractometer at 25.0°C with water bath.

Discard first cut (FI) of 121.5 mL (27 min) and collect fraction (FII) of next 67.5 mL (27–42 min) in round-bottom flask. Rotary-evaporate CAREFULLY as above just to dryness and dissolve residue in 3.0 mL acetonitrile–methanol (1 + 1) for LC. For tissues containing low levels of PAHs, add 1.0 mL 0.2% (w/v) paraffin oil in methanol–chloroform (1 + 1) as "keeper" to FII and rotary-evaporate as above. Transfer residue to urinary sediment tube or Shevky-Stafford albumin tube, using three 0.5 mL rinses of methanol–chloroform (1 + 1, v/v), and evaporate solvent under stream of nitrogen. Warming tube to 35°C in water bath is permitted but extended periods of time under nitrogen stream is not recommended. Cool tube to room temperature and dilute solution to suitable volume (100–250 μL) with methanol–chloroform for injection into LC apparatus. Use *gentle agitation* to dissolve residue so as not to change solvent composition by evaporation because this will result in incomplete dissolution of paraffin oil "keeper." Transfer to limited volume insert for use in Waters WISP injector or inject ca 50 μL manually.

Liquid Chromatography

Elute individual PAHs with solvent A (acetonitrile–water (40 + 60)) and solvent B (acetonitrile) with the following gradient (v/v):

Elapsed time, min	*Flow, mL/min*	*A, %*	*B, %*	*Curve*
0	1.0	80	20	
18	1.5	5	95	06
20	1.5	1.5	98.5	01
30	1.5	1.5	98.5	01
35	1.0	80	20	01

Table 1. Recoveries of PAHs added to 2 g lobster digestive gland homogenate[a]

PAH	Added, μg	Mean rec., %	RSD, % ($N=3$)
Fluoranthene	3.4	105.4	8.3
Pyrene	8.2	87.7	1.4
Benz[*a*]anthracene	1.7	112.4	9.4
Chrysene	7.2	94.0	4.4
Benzo[*b*]fluoranthene	3.4	108.9	3.8
Benzo[*k*]fluoranthene	1.7	108.5	0.0
Benzo[*a*]pyrene	1.7	99.1	6.8
Dibenz[*a,h*]anthracene	3.4	104.6	3.6
Benzo[*ghi*]perylene	3.4	88.8	6.9
Indeno[1,2,3-*cd*]pyrene	1.7	104.6	6.0

[a]LC, fluorescence detection.

Table 2. Recoveries of PAHs added to 2 g blue mussel homogenate following overnight exposure to alcoholic potassium hydroxide solution[a]

PAH	Added, ng	Mean rec., %	RSD, % ($N=3$)
Fluoranthene	2.4	96.2	4.9
Pyrene	13.0	92.1	14.4
Benz[*a*]anthracene	1.1	94.7	8.6
Chrysene	12.0	91.8	12.0
Benzo[*e*]pyrene	11.0	83.0	0.0
Benzo[*b*]fluoranthene	1.0	94.0	3.1
Benzo[*k*]fluoranthene	0.5	96.5	11.1
Benzo[*a*]pyrene	1.3	89.6	10.0
Benzo[*ghi*]perylene	7.6	75.3	16.9
Indeno[1,2,3-*cd*]pyrene	5.0	86.1	16.3

[a]LC, fluorescence detection.

Results and Discussion

Saponification and Extraction

The use of simple, easily cleaned glassware results in considerable savings of time and money. When small tissue samples are processed and saponification is complete, problem emulsions are eliminated. Also, with minimal glassware and small quantities of reagents, blank values are insignificant (Figure 2).

Gel Permeation Chromatography

Separation of PAHs from biogenic interferences by GPC with Sephadex and μStyragel has been reported (1, 4). A short technical note (6) published in 1979 by the Autoprep 1001 manufacturer indicated that Bio-Beads could be used in this application as well but, to date, the use of this gel for isolation of PAHs has not been fully exploited.

Figure 3 shows the type of separation of PAHs from lipoid interferences obtained under our experimental conditions. Curves A, B, C, and D are gel permeation-UV absorption chromatograms of extracts from saponified digestive glands of lobsters captured in the vicinity of a coking plant at Sydney Harbour, Nova Scotia, Canada; curve A represents animals captured at some distance from the plant, whereas curve D represents animals captured much closer to the plant. Curve E is a procedural blank determination. Each sample was composed of pools of equal weights of 5 individual homogenized glands. Fraction I (FI) is the nonsaponifiable fraction and is discarded. Fraction II (FII) is the PAH fraction. The separation is very good, yielding a PAH fraction which can be injected into the LC column without further cleanup. Curve F is the refractive index (RI) response equivalent of curve D. RI is much less sensitive to PAHs than is UV but is useful for monitoring the elution of lipid from the column and for indicating whether any major potentially interfering lipoid material coelutes with the PAHs.

FII of curves A, B, C, D, and E were collected and rechromatographed by LC with fluorometric detection. Figure 2 shows the chromatogram obtained from a typical lobster hepatopancreas in which the following PAHs were measured (in order of elution): fluoranthene, pyrene, benz[*a*]anthracene, chrysene, benzo[*e*]pyrene, benzo[*b*]fluoranthene, benzo[*k*]fluoranthene, benzo[*a*]pyrene, benzo[*ghi*]perylene, and indeno [1,2,3-*cd*]pyrene. These accounted for the major fluorescent components present in the samples. (Dibenz[*a,h*]anthracene was not quantified in the lobster samples but is shown for purposes of illustration.) The concentrations of the PAHs in the Sydney Harbour lobster hepatopancreas were summed and plotted against GPC-UV responses ($r^2 = 0.9997$). Good linearity of response was obtained over 4 orders of magnitude. The summed PAH concentrations are also shown beside curves A, B, C, and D of Figure 3.

Recoveries of PAHs added before saponification are given in Tables 1 and 2 (microgram and nanogram amounts of added PAHs, respectively). For recovery studies, the analyses were performed in triplicate. In general, the recoveries of all PAHs listed were quantitative and of acceptable precision. To ensure that recoveries of PAHs were not adversely affected by the overnight exposure to the alcoholic potassium hydroxide solution, the data in Table 2 were obtained from samples of blue mussel tissue analyzed, after spiking, by the overnight room temperature tissue dissolution procedure. Initially, problems of erratic recoveries (40–85%) were experienced at low levels of added PAH and it was suspected that these problems were due to volatilization losses. The difficulty was overcome by addition of a "keeper" (1 mL 0.2% paraffin oil in methanol–chloroform (1 + 1)) to the solution from the GPC step before rotary evaporation. While this worked well, it necessitated switching to a methanol–chloroform solvent mixture for dissolution before LC analysis. Special precaution had to be observed when making up the final 100 μL solution for LC injection, i.e., very gentle agitation was used so as not to change the solvent ratio by evaporation during dissolution. It should also be noted that injection of large volumes (50 μL) into the chromatograph changes the peak shapes of the early eluters (fluoranthene, pyrene, and benz[*a*]anthracene) and it is necessary to inject the same volumes of samples and standard solutions to obtain proper quantitation (peak height) at the 0.5–10 ng PAH level.

Various investigators have reported low and/or erratic recoveries of several PAHs, including the important carcinogen, benzo[*a*]pyrene, following the use of cleanup adsorbents such as Florisil and silica gel (2, 4, 5, 7). In our procedure this problem is eliminated.

The possibility of interference by polychlorinated biphenyls (PCB) in the GPC-UV procedure should not be overlooked. This interference should be very small except in cases of very heavy PCB contamination because most chlorobiphenyls absorb maximally at 200–220 nm whereas the wavelength used in the GPC-UV is 254 nm, a region of the spectrum where PCB absorbs weakly (8). As an environmental contaminant or as the dehydrochlorinated product of *p,p'*-DDT formed during saponification, *p,p'*-DDE could also interfere if present in high enough concentrations (λ(max) = 247 nm). However, previous unpublished work on lobsters from the area under study indicated that the concentrations of organochlorines were 1–2 orders of magnitude lower than the PAH concentrations (e.g., 2–3 μg total PCB/g (wet weight) and 0.3–0.5 μg *p,p'*-DDE/g (wet weight) in lobster digestive glands) and therefore would not present a problem. In addition, no

significant unexplainable peaks were seen in our samples by LC-UV analysis under the chromatographic conditions used. Also, further work indicated that the GPC-UV absorption wavelength could be set at 280 nm to discriminate further against potential interference by such contaminants.

The possibility of detection of other classes of compounds such as alkyl PAHs, arising from sources such as crude petroleum and refined petroleum products, by the GPC-UV method should be recognized. While the GPC-UV method could obviously be used to give an estimate of the concentrations of such compounds, the analyst should recognize that other analytical procedures (e.g., GC-MS) in addition to the simple LC procedure following GPC-UV may be required to fully characterize such analytes.

The Bio-Beads SX-3 column can be used for many hundreds of samples without apparent deterioration; occasionally it may be necessary to replace approximately the first 0.5 cm of packing after prolonged use in processing samples containing relatively large amounts of nonsaponifiable material.

Conclusions

The GPC-UV method described seems ideal as a screening method for rapid estimation of the extent of PAH contamination of marine biota in situations such as harbors and could be used to quickly scan areas impacted by oil spills. Further quantitative information on individual PAHs can be obtained by LC. The use of small tissue samples permits LC analysis without cleanup other than by GPC, thus eliminating many of the problems associated with analysis of the more labile and/or volatile compounds. The use of small quantities of reagents and simple, easily cleaned glassware results in undetectable procedural blanks. GPC and LC procedures are automated so that approximately 50 analyses can be carried out by 2 analysts in 1 week. The GPC-UV procedure can be optimized for a particular PAH and the results expressed in the popular "chrysene or pyrene equivalents" (9). Recovery studies of very low levels of PAHs indicate that the method is quantitative if proper precautions are taken.

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Determination of Halogenated Phenols in Raw and Potable Water by Selected Ion Gas Chromatography-Mass Spectrometry

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Pentafluorobenzylation and in situ acetylation are compared in the determination of phenol and halogenated phenols in water samples. The latter technique is considered superior to the former for determining phenols at the ng/L level because of less background interference and better recoveries (80% or better except for pentachlorophenol and trichloroguaiacol which had recoveries of about 60%). Further evaluation of the in situ technique by electron capture gas chromatography and gas chromatography-mass spectrometry shows that the latter, in the selected ion monitoring mode, is more suitable because, unlike GC-ECD, it can confirm and quantitate all phenols. In particular, GC-ECD could not detect even high levels of phenol and the monohalogenated phenols. Phenols at 5–473 ng/L were detected in some Canadian drinking water supplies by the in situ acetylation technique combined with GC-MS.

Chlorophenols have been frequently reported in surface waters (1–3), sediments (2, 4, 5), and municipal and industrial discharges (6–8) and are known to cause taste and odor problems in drinking water (9). It has also been reported that bromination of phenols can occur in water from the reaction of phenol with chlorine in the presence of bromide ion (10, 11). In preparation for a national survey of Canadian drinking water, in which levels of chloro- and bromophenols were to be determined, it was necessary to validate appropriate analytical methodology, particularly for the bromophenols.

Common methods for determining chlorophenols at trace levels are based primarily on chromatographic techniques; gas chromatography (GC) methods (1, 12–21) predominate over liquid chromatography methods (22, 23). The GC methods include analysis of derivatized and underivatized phenols. Because of the instability and tailing of underivatized phenols and their chemical activity within the injection port and column, several derivatization procedures have been used to improve the chromatography and to enhance sensitivity by employing derivatives with functional groups which are amenable to specific detection in the electron capture mode (14–16).

Literature reports (2) concentrate mainly on detecting chlorophenols and, occasionally, chloroguaiacols in the environment; of these methods, 2 derivatization procedures, in situ acetylation (1, 17–21) and formation of the pentafluorobenzyl (PFB) derivative (15, 24), were initially compared for determining chloro- and bromophenols in raw and potable water samples. Some problems which prevented the use of the PFB derivative are discussed. Usefulness of the acetate deriva-

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Table 1. GC and GC/MS data for some halogenated phenol acetates

Phenol acetate	Compound	RRT[a]	Detection limit, pg		GC-MS	
			ECD	GS-MS-SIM	Selected ions	
Phenol	1	5.28	—	10	94.05	136.05
2-Chloro	2	6.60	200	10	128.05	129.95
3-Chloro	3	6.86	200	10	128.05	129.95
4-Chloro	4	6.90	200	10	128.05	129.95
2-Bromo	5	7.23	200	5	171.90	173.90
4-Bromo	6	7.55	200	5	171.90	173.90
2,6-Dichloro	7	7.57	20	5	161.95	163.95
2,4-Dichloro	8	7.74	20	5	161.95	163.95
2,5-Dichloro	9	7.74	20	5	161.95	163.95
3,5-Dichloro	10	7.85	20	5	161.95	163.95
2,3-Dichloro	11	7.95	20	5	161.95	163.95
2-Chloro-6-bromo	12	8.14	10	10	205.85	207.87
3,4-Dichloro	13	8.15	20	5	161.95	163.95
2-Bromo-4-chloro	14	8.31	10	10	205.85	207.85
2-Chloro-4-bromo	15	8.37	10	10	205.85	207.85
2,4,6-Trichloro	16	8.41	10	20	195.95	197.95
2,3,6-Trichloro	17	8.63	10	20	195.95	197.95
2,6-Dibromo	18	8.67	10	5	249.90	251.90
2,3,4-Trichloro	19	8.73	10	20	195.95	197.95
2,3,5-Trichloro	20	8.77	10	20	195.95	197.95
2,4-Dibromo	21	8.86	20	5	249.90	251.90
6-Bromo-2,4-dichloro	22	8.92	10	5	241.85	243.85
4-Bromo-2,6-dichloro	23	8.96	10	5	241.85	243.85
2,3,4-Trichloro	24	9.00	10	20	195.95	197.95
3,4,5-Trichloro	25	9.11	10	20	195.95	197.95
4-Chloro-2,6-dibromo	26	9.45	10	10	285.85	287.85
2-Chloro-4,6-dibromo	27	9.50	10	10	285.85	287.85
2,3,4,6-Tetrachloro	28	9.51	7	5	229.85	231.95
2,3,5,6-Tetrachloro	29	9.51	7	5	229.85	231.95
2,3,4,5-Tetrachloro	30	9.85	7	5	229.85	231.95
2,4,6-Tribromo	31	10.00	10	5	329.80	331.80
2,4,6-Tribromo-C^{13}	32	10.00	10	5	335.80	337.80
3,4,5-Trichloroguaiacol	33	10.09	10	5	225.85	227.85
2,3,4,5,6-Pentachloro	34	10.48	5	10	263.80	265.85
2,3,4,5-Tetrachloroguaiacol	35	10.59	7	10	261.80	263.80

[a]Retention time relative to ^{13}C-2,4,6-tribromophenol acetate (RRT = 10.00) = 14.63 min on 25 m × 0.31 mm id DB-1 column.

Table 2. GC data and recoveries of chlorophenols as PFB derivatives

Derivative	Compound	RRT[a]	Fortification[b]	Recovery,[c] %		
				Raw	Treated	Distilled
Phenol	1	3.14	60	58 ± 56	64 ± 29	10 ± 2
2-Chloro	2	3.96	85	71 ± 18	59 ± 4	41 ± 21
4-Chloro	4	4.29	52	42 ± 19	46 ± 17	25 ± 15
2,6-Dichloro	7	4.75	59	86 ± 9	66 ± 1	59 ± 16
2,4-Dichloro	8	5.43	49	104 ± 9	71 ± 5	65 ± 16
2,4,6-Trichloro	16	6.01	57	108 ± 10	79 ± 4	72 ± 18
2,4,5-Trichloro	20	7.03	74	116 ± 9	80 ± 4	79 ± 16
2,3,4,6-Tetrachloro	28	8.04	64	117 ± 9	80 ± 5	88 ± 13
2,3,4,5-Tetrachloro	30	9.23	73	129 ± 9	81 ± 10	92 ± 14
2,3,4,5,6-Pentachloro	34	10.00	91	124 ± 10	93 ± 5	121 ± 16

[a]Retention time relative to pentachlorophenol PFB derivative (RRT = 10.00) = 25 min on 15 m × 0.32 mm id DB-5 capillary column.
[b]ng Phenol/800 mL water.
[c]Mean ± standard deviation for 5 replicate samples.

tives was compared by gas chromatography with electron capture detection (GC-ECD) and gas chromatography–mass spectrometry (GC-MS) using selected ion monitoring (SIM); the advantages and disadvantages of the 2 techniques are discussed.

Experimental

Apparatus

(a) *Gas chromatographs.—(i) For acetates:* Perkin Elmer Model 910, modified for capillary column operation, equipped with ^{63}Ni electron capture detector and Model 4000 Spectra Physics integrator. Column parameters and operating conditions: 30 m × 0.25 mm id DB-1 (J & W) fused silica capillary column. Using syringe (Hamilton, Model 701SN) with 3 in. needle, introduce 1 μL aliquot through splitless injector system (SGE); open vent after 60 s. Temperatures: injector 260°C; detector 320°C; column oven program: initial 80°C, hold 1 min, program at 10°/min to 220°C, hold 2 min, post-program 265°C, hold 4 min. Helium carrier gas flow 1.2 mL/min, and nitrogen make-up gas flow 48 mL/min.

(ii) For pentafluorobenzyl derivatives: Hewlett-Packard Model 5880A with ^{63}Ni electron capture detector (ECD). Column parameters and operating conditions: 15 m × 0.32 mm id DB-5 fused capillary, hydrogen carrier gas at 6 psi with 5% methane in argon make-up gas at 30 mL/min. Introduce 3.8 μL aliquot through splitless injector; open vent after 0.45 min. Temperatures: injector 220°C; detector 330°C; column oven program: initial 60°C, hold 3 min, program at 10°/min to 110°C and then at 2.5°/min to 200°C.

(b) *Gas chromatograph–mass spectrometer (GC-MS).*—Hewlett-Packard Model 5992B gas chromatograph–mass spectrometer with Model 9825A on-line data system and 2 Model 9885S disk drives. Operating conditions: 25 m × 0.31

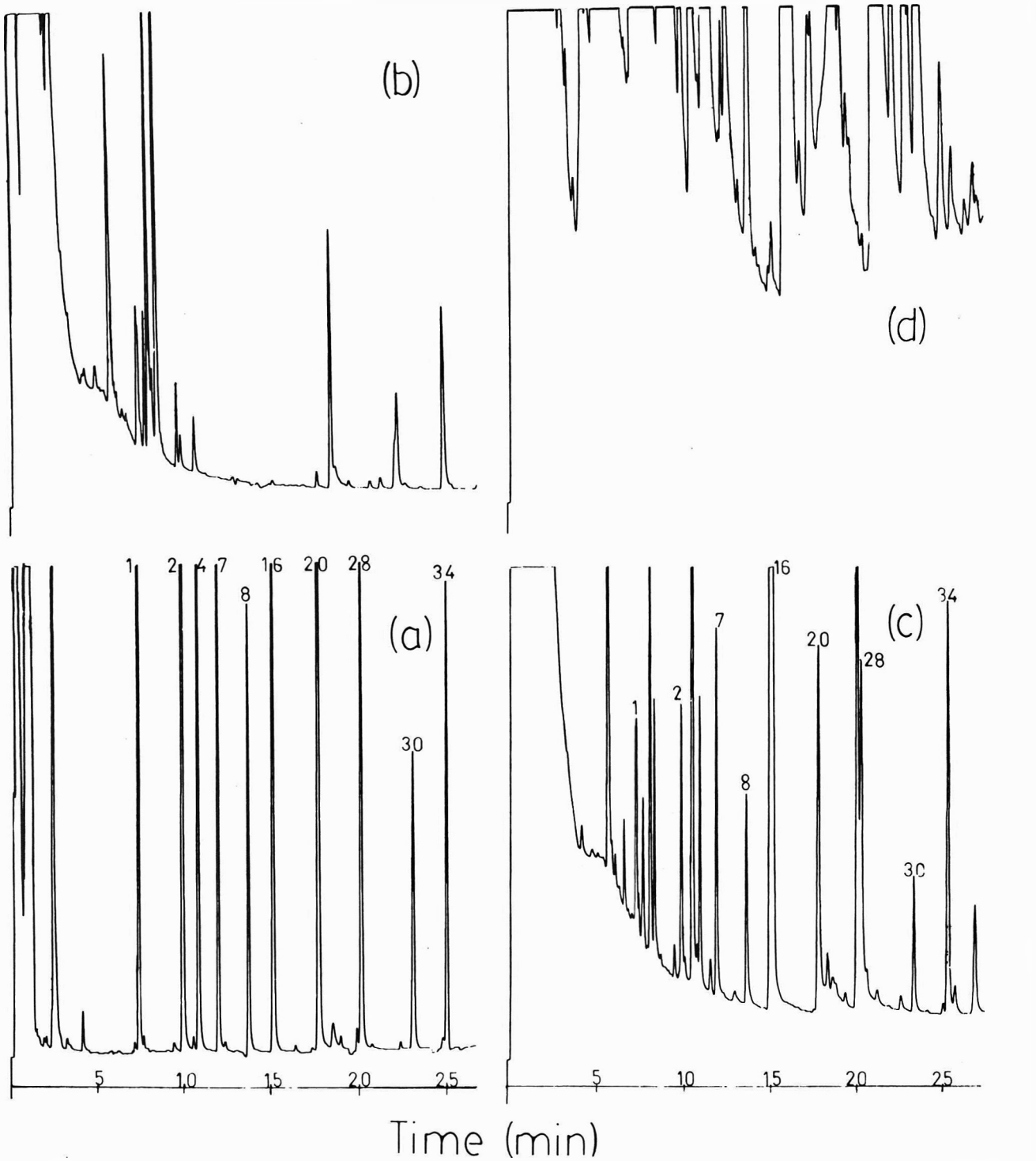

Figure 1. Pentafluorobenzyl derivatives of phenol and halogenated phenols: (a) Chromatogram of standard mixture; (b) chromatogram of reagent blank after silica column cleanup; (c) chromatogram of fortified water sample (49–91 ng/L) collected in February 1984; (d) chromatogram of fortified water sample collected from same site in July 1984. See Table 1 for peak identification.

mm id DB-1 (J & W) fused silica capillary; helium carrier gas at 1.5 mL/min; 2 μL splitless injections with splitter opened after 60 s. Temperatures: injector 240°C; initial oven 50°C, hold 4.6 min, program at 15°/min to 260°C. Operate mass spectrometer in selected ion mode (SIM) with dwell times of 20 ms/ion. Glass-lined open split interface/restrictor as GC-MS interface with flow rate ca 0.8 mL/min entering MS instrument. Calibrate GC-MS system each day, using instrument autotune parameters.

(c) *Glassware.*—Soak in acid dichromate solution, rinse with water, and wash with acetone before use.

Reagents

(a) *Solvents.*—Hexane and methylene chloride (CH_2Cl_2); glass-distilled quality (Caledon Laboratories, Georgetown, Ontario, Canada). Distill twice in all-glass apparatus.

(b) *Purified water.*—Distill Super-Q water over acidic potassium permanganate (1 mg $KMnO_4$ + 0.5 mL conc. H_2SO_4/L water). Acidify distilled water (4 L) to pH 2, extract with 50 mL CH_2Cl_2, make basic (pH 9) with 30% NaOH solution, and add 5 mL acetic anhydride. Shake 2 min and extract water with hexane (2 × 50 mL). Use water for spiking studies and for preparation of NaOH solution.

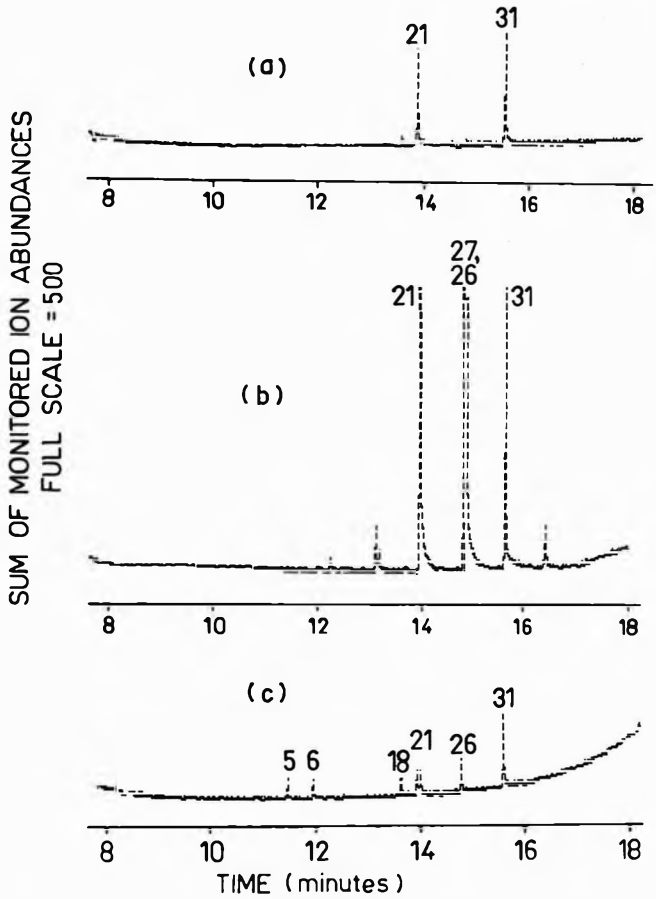

Figure 2. In situ acetylation of phenol and halogenated phenols: (a) GC-MS chromatogram of reagent blank with interferences; (b) GC-MS chromatogram of acetylated rubber bulb extract; (c) GC-MS chromatogram of a standard mixture (400 pg) monitored for brominated phenols. See Table 1 for peak identification.

(c) *Anhydrous sodium sulfate.*—Wash granular, reagent grade material with acetone, hexane, and CH_2Cl_2. Dry and heat overnight at 700°C. Cool and store in a glass bottle with Teflon-lined cap.

(d) *NaOH solution.*—30% w/v AnalaR grade in purified water. Add 30 mL acetic anhydride to 2 L 30% NaOH solution, shake 2 min, and extract with hexane (2 × 50 mL). Store purified NaOH solution in dispensing bottle equipped with Florisil guard tube to filter incoming air.

(e) *Acetic anhydride.*—Distill twice at 139.5°C. Store in dispensing bottle equipped with Florisil guard tube to filter incoming air.

(f) *Phenols.*—Obtained from various manufacturers. Those obtained in bulk were purified by re-crystallization or distillation. Prepare 2,6-dibromo-4-chlorophenol by in situ bromination of 2-bromo-4-chlorophenol as follows: To 50 mg phenol in 100 mL water in 125 mL separatory funnel, add 20 mL bromate/bromide solution (1 g KBr + 0.28 g $KBrO_3$ in 100 mL water) followed by addition of 2 mL concentrated HCl; shake mixture vigorously. After 10 min, add 3 mL 0.1N sodium thiosulfate to destroy excess bromine. Extract dibromochlorophenol into 5 mL hexane, dry over sodium sulfate, and evaporate to dryness. Similarly, synthesize ^{13}C-2,4,6-tribromophenol by in situ bromination of ^{13}C-phenol (Merck Frost, Montréal, Canada).

(g) *Acetates.*—Macro-scale syntheses were performed on parent phenols according to the method of Chau and Coburn (25). The identity of the acetates was confirmed by GC-MS.

(h) *Stock solutions.*—Prepare 100 ng/mL phenol mixture in acetone. Prepare acetate standards in hexane by serial dilution of a stock 10 μg/mL solution.

Collection and Preservation of Samples

Collect 800 mL raw and treated water in 1 L precleaned amber bottles with Teflon-lined caps.

Preserve 800 mL purified, raw, treated, or fortified water samples by addition of 80 mg sodium thiosulfate, 3.0 mL 50% sulfuric acid, and 50 mL CH_2Cl_2, and store at 4°C.

In Situ Acetylation

Add 100 ng ^{13}C-2,4,6,-tribromophenol in 10 μL acetone to 800 mL preserved samples in 1 L sample bottle and then add 7 mL 30% NaOH solution and gently shake. Transfer to separatory funnel, shake vigorously for 2 min, let layers separate, and discard CH_2Cl_2 layer. Add acetic anhydride (7 mL) and shake vigorously ca 20 s. Extract by shaking with 4 mL hexane ca 2 min, dry hexane layer by passage through column of sodium sulfate. Rinse sides of separatory funnel and sodium sulfate column. Evaporate under gentle stream of pure nitrogen to 1 mL for GC-ECD analysis or to 0.3 mL for GC-MS-SIM analysis.

Pentafluorobenzylation of Chlorophenols

Extract phenols with CH_2Cl_2, evaporate CH_2Cl_2 and exchange to acetone, add pentafluorobenzyl bromide, and

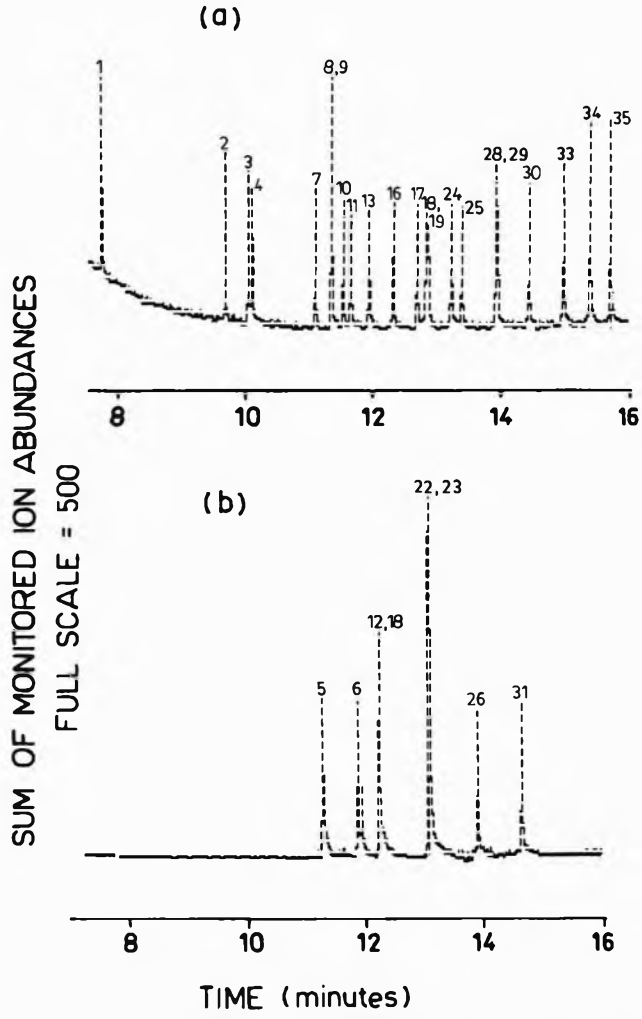

Figure 3. Representative chromatograms of acetylated phenol and halogenated phenols monitored by GC-MS in SIM mode: (a) monitoring of phenol, chlorophenols, and chloroguaiacols; (b) monitoring of brominated phenols and chlorophenols. See Table 1 for peak identification.

Table 3. Recoveries of halophenols (ng/L) from fortified water

Compound	Raw water[a]			Treated water[a]		
	25	100	500	25	100	500
Phenol	105 ± 20	95 ± 15	93 ± 14	132 ± 12	100 ± 19	70 ± 10
2-Chlorophenol	107 ± 20	134 ± 20	110 ± 10	125 ± 13	109 ± 17	86 ± 16
4-Chlorophenol	122 ± 20	105 ± 10	90 ± 7	84 ± 14	105 ± 19	101 ± 4
2,6-Dichlorophenol	99 ± 20	87 ± 12	96 ± 10	113 ± 11	124 ± 15	94 ± 5
2,4-Dichlorophenol	95 ± 9	115 ± 11	102 ± 6	84 ± 14	98 ± 13	101 ± 5
2,4,6-Trichlorophenol	88 ± 13	101 ± 10	105 ± 6	104 ± 12	100 ± 17	109 ± 3
2,4,5-Trichlorophenol	107 ± 7	122 ± 4	93 ± 8	85 ± 14	116 ± 11	102 ± 6
2,3,4,6-Tetrachlorophenol	86 ± 14	85 ± 8	73 ± 5	103 ± 19	89 ± 15	118 ± 1
2,3,4,5-Tetrachlorophenol	77 ± 16	124 ± 4	106 ± 11	113 ± 4	123 ± 14	102 ± 11
Pentachlorophenol	56 ± 18	66 ± 9	68 ± 3	54 ± 15	70 ± 13	76 ± 1
3,4,5-Trichloroguaiacol	56 ± 21	61 ± 2	80 ± 4	61 ± 26	52 ± 8	72 ± 9
Tetrachloroguaiacol	77 ± 20	75 ± 14	92 ± 4	71 ± 22	77 ± 21	83 ± 4
2-Bromophenol	99 ± 20	81 ± 4	92 ± 4	109 ± 29	102 ± 9	92 ± 8
4-Bromophenol	105 ± 26	89 ± 4	89 ± 2	117 ± 30	106 ± 11	89 ± 9
2,6-Dibromophenol	91 ± 13	113 ± 4	87 ± 2	97 ± 25	104 ± 9	89 ± 3
2,4-Dibromophenol	85 ± 19	102 ± 18	100 ± 8	125 ± 16	77 ± 7	113 ± 6
4-Bromo-2,6-dichlorophenol	76 ± 10	73 ± 4	111 ± 5	68 ± 23	65 ± 6	108 ± 9
2,4,6-Tribromophenol	92 ± 13	81 ± 11	92 ± 2	102 ± 12	89 ± 6	98 ± 1

[a]% Recovery ± standard deviation; 4 samples.

Table 4. Recoveries of halophenols from fortified field samples

Compound	Raw water[a]	Treated water[a]
Phenol	118 ± 17	109 ± 20
2-Chlorophenol	105 ± 20	109 ± 25
4-Chlorophenol	104 ± 20	115 ± 24
2,6-Dichlorophenol	115 ± 20	109 ± 24
2,4-Dichlorophenol	120 ± 9	101 ± 13
2,4,6-Trichlorophenol	96 ± 10	100 ± 15
2,4,5-Trichlorophenol	87 ± 3	86 ± 12
2,3,4,6-Tetrachlorophenol	56 ± 7	43 ± 10
2,3,4,5-Tetrachlorophenol	83 ± 10	71 ± 5
Pentachlorophenol	37 ± 15	29 ± 17
3,4,5-Trichloroguaiacol	63 ± 5	64 ± 10
Tetrachloroguaiacol	51 ± 20	47 ± 24
2-Bromophenol	96 ± 5	101 ± 10
4-Bromophenol	109 ± 6	125 ± 11
2,6-Dibromophenol	73 ± 4	79 ± 6
2,4-Dibromophenol	111 ± 5	149 ± 25
4-Bromo-2,6-dichlorophenol	96 ± 15	124 ± 15
2,4,6-Tribromophenol	35 ± 10	33 ± 6

[a]Fortified at 100 ng/800 mL water; % recovery ± standard deviation; 8 samples.

heat at 60°C for 30 min, as in previously published methods (15, 23). Use 3-stage evaporation procedure: *(i)* 170 to 35 mL in 250 mL round-bottom flasks; *(ii)* 35 mL (plus 3 × 3 mL acetone washings) to 8 mL in 50 mL round-bottom flasks; *(iii)* 8 mL (plus 3 × 2 mL acetone washings) to 2 mL in 15 mL glass centrifuge tubes under gentle stream of nitrogen. Use rotary evaporator with a water bath at room temperature and low vacuum for the volume reduction, taking necessary precautions to avoid bumping. Total evaporation time: ca 1 h.

Silica column cleanup was carried out as previously reported (15).

Gas Chromatography

Inject aliquot of concentrated extract into GC apparatus and quantitatively determine amount of unknown or fortified material by comparing its peak height or area with that of corresponding standard injected under similar conditions.

Gas Chromatography–Mass Spectrometry

Inject aliquot into GC-MS apparatus. Monitor each compound by selected ion monitoring using 2 ions per compound (Table 1). Quantitate by comparing peak heights of the 2 characteristic ions with corresponding peak heights for a standard injected under similar conditions.

Results and Discussion

The PFB method (15, 23) gave promising results during initial evaluation because of the sensitivity of the ECD detector to the PFB derivatives. Quantitation limits were ca 30–40 pg injected for each compound which, for a 800 mL water sample, would represent 20–30 ng chlorophenol/L. A standard chromatogram is shown in Figure 1a. Chromatograms with only a few interferences were obtained for the reagent blank after silica column cleanup (Figure 1b). Fortification studies on raw, treated, and Super-Q water at ca 50–100 ng/L gave recoveries greater than 65% for all chlorophenols except for the monochlorophenols and phenol itself (Table 2). As discussed by Lee et al. (15), considerable care needs to be taken to achieve good recoveries of chlorophenols during evaporation of the extraction solvent. Analysis of water samples collected at a potable water treatment plant in February 1984 gave acceptable chromatograms in which chlorophenols fortified at 49–91 ng/L could easily be detected (Figure 1c). However, analysis of water samples collected at the same treatment plant in July gave chromatograms which showed massive interferences (Figure 1d) which could not be significantly reduced by silica column cleanup. These interferences were still present in water samples collected several weeks later in August and occurred both in raw and treated water but not necessarily in both at the same time. It is probable that this resulted from intake water quality variations and the water retention time in the treatment plant. Analysis of these water samples by GC-ECD using the in situ acetylation method indicated that these interferences were not chlorophenols. Although the identity of these interferences could not readily be ascertained it was decided that the PFB method would not be suitable for use in our proposed national survey.

Further method evaluation was, therefore, restricted to the in situ acetylation method which has been used by a number of workers for determining chlorophenols in various types of waters (1, 17–21). The method is relatively straightforward and the acetylated phenols can be easily extracted into a small volume of solvent so that losses in subsequent solvent evaporation steps are minimized. It also has the advantage that nonacidic compounds can be removed from the water sample by solvent extraction of the base solution before acetylation. This results in cleaner extracts for GC analysis. However, there are difficulties in purifying the blank water and reagents so that an acceptable method blank can be obtained for the

Table 5. Halogenated phenol concentrations in raw and treated water

City	Water type	Phenol	Halogenated phenols, ng/L[a]									
			2-Cl	4-Cl	2,4-diCl	2,4,6-triCl	Penta-Cl	2-Br	2,6-diBr	2,4,6-triBr	4-Br-2,6-diCl	4-Cl-2,6-diBr
1	R[b]	448 ± 105	ND[c]	ND	ND	ND	34 ± 5	ND	ND	ND	ND	ND
	T[d]	NQ[e]	ND	34 ± 5	9 ± 1	16 ± 2.8	5 ± 0.8	ND	ND	ND	6 ± 3	ND
2	R	NQ	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	T	NQ	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
3	R	NQ	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	T	NQ	ND	ND	ND	ND	ND	ND	ND	5 ± 0.3	8 ± 1	8 ± 2
4	R	NQ	ND	ND	ND	12 ± 2[f]	ND	ND	ND	ND	ND	ND
	T	NQ	ND	5 ± 1	5 ± 1	19 ± 3	ND	ND	ND	ND	ND	ND
5	R	NQ	ND	ND	ND	ND	ND	ND	ND	ND	12 ± 2	5 ± 2
	T	NQ	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
6	R	48 ± 4	ND	15 ± 0.4	ND	ND	5 ± 1	ND	ND	ND	ND	ND
	T	208 ± 34	39 ± 3	32 ± 2	17 ± 1	60 ± 1	ND	42 ± 3	60 ± 2	ND	42 ± 5	8 ± 1

[a]Mean ± standard deviation; 2 ions for each of duplicate samples.
[b]Raw water.
[c]Not detected.
[d]Treated water.
[e]Not quantified; phenol peak present but less than twice blank value.
[f]Mean ± standard deviation; 2 ions for a single sample.

low ng/L range. Simple solvent extraction of the redistilled water was not adequate and, therefore, a more complicated procedure involving acetylation of the impurities was used. Despite all efforts, a small amount of phenol, equivalent to 23 ± 5 ng/L, was still present in the method blank. One significant potential source of interference for the bromophenols was the rubber bulb used with Pasteur pipets for transferring small volumes of solution. Although these bulbs do not come in direct contact with the solutions, considerable interferences (Figure 2a) randomly occurred until these bulbs were discarded and any required transfers were made with a stainless steel/glass syringe. The interferences had similar m/z values and retention times as the standards. However, CH_2Cl_2 washing of a base extraction of the bulbs showed that the interferences were not bromophenols because they did not remain in the aqueous phase. Therefore, the interferences would not be a problem in field samples because they will not go through the whole extraction procedure.

The extracted acetates can be analyzed by GC-ECD (17–19, 21) or by GC-MS-SIM (21, 26); both techniques have advantages and disadvantages. The ECD response factors for the acetylated phenols increased with increase in the number of halogen atoms present, were poor for the mono-substituted phenols, and were essentially zero for phenol itself at ng/L levels. The minimum injected amount which could be detected in field samples is indicated in Table 1 together with the retention times of the halogenated phenols and guaiacols investigated. The sensitivity of ECD could not be fully exploited because of background response. GC-ECD can also be subject to interferences that can be misidentified as chlorophenols (26). GC-MS-SIM has higher detection limits for the more halogenated phenols but is much more sensitive than ECD for phenol and the monohalogenated phenols. It is also less subject to interferences and, by monitoring 2 ions per compound, provides stronger confirmation of the identity of the phenols. By appropriate choice of ions, compounds with similar retention times can easily be distinguished. However, because of this selectivity, other compounds present in the sample are not likely to be detected and potentially significant information may be missed. For the determination of chlorophenols in drinking water, however, it is essential to be able to monitor the less halogenated phenols because these are known to be formed during disinfection with chlorine in the water treatment process (9). Therefore, GC-MS-SIM was selected as the analytical technique of choice for monitoring halogenated phenols in drinking water. For each group of phenols 2 characteristic ions (Table 1) were selected from the mass spectral fragmentation patterns of the phenol acetates and these ions were monitored during the GC-MS-SIM analysis. Since the available data system was limited to monitoring 20 ions/run, 2 GC-MS-SIM analyses had to be made per sample. In the first injection, phenol, chlorophenols, and the guaiacols were monitored; in the second injection, the bromophenols and bromochlorophenols were monitored. The sensitivity of this technique depends on the signal-to-noise ratio which depends in part on the number of ions monitored during an analysis and their respective dwell times. The detection limits for 20 ions monitored/run with a dwell time of 20 ms/ion are listed in Table 1. The detection limits for the trichlorophenols are somewhat poorer than for the other phenols because of a higher background for the 2 ions monitored. A linear response was obtained in the SIM mode for all of the acetates over the range of 5 to 1000 pg injected. This would be equivalent to a concentration range of about 1 ng/L to 2 μg/L in the original water sample for all phenols except the trichlorophenols for which the range would be 5 ng/L to 2 μg/L.

Several capillary columns of different polarity were evaluated but no single column could completely resolve all of the compounds investigated. A DB-1 column was found to give the best overall resolution of the halogenated phenol acetates; their retention times on this column are listed in Table 1 and representative chromatograms are shown in Figures 3a and 3b. Compounds with similar retention times but with different molecular weights, i.e., 6/7, 12/13, 17/18, and 27/28, could be easily differentiated by selected ion monitoring. Those compounds with the same molecular weight and similar retention times, however, could not be differentiated, i.e., 8/9, 19/20, 22/23, 26/27, and 28/29.

Before acetylation, each sample was fortified with ^{13}C-2,4,6-tribromophenol (equivalent to 100 ng/800 mL) to verify that the acetylation, extraction, and concentration steps were acceptable. The recovery of the ^{13}C-compound was 98.2 ± 10%.

The percent recoveries of phenols from water samples fortified at 25, 100, and 500 ng/800 mL were 80% or better except for pentachorophenol and trichloroguaiacol, which had somewhat lower recoveries (Table 3). Analytical precision in the fortification studies was usually better than 15, 20, and 30% at the 500, 100, and 25 ng/800 mL levels, respectively (Table 3). Field samples fortified at the water treatment plant at 100 ng/800 mL and transported back to the laboratory gave

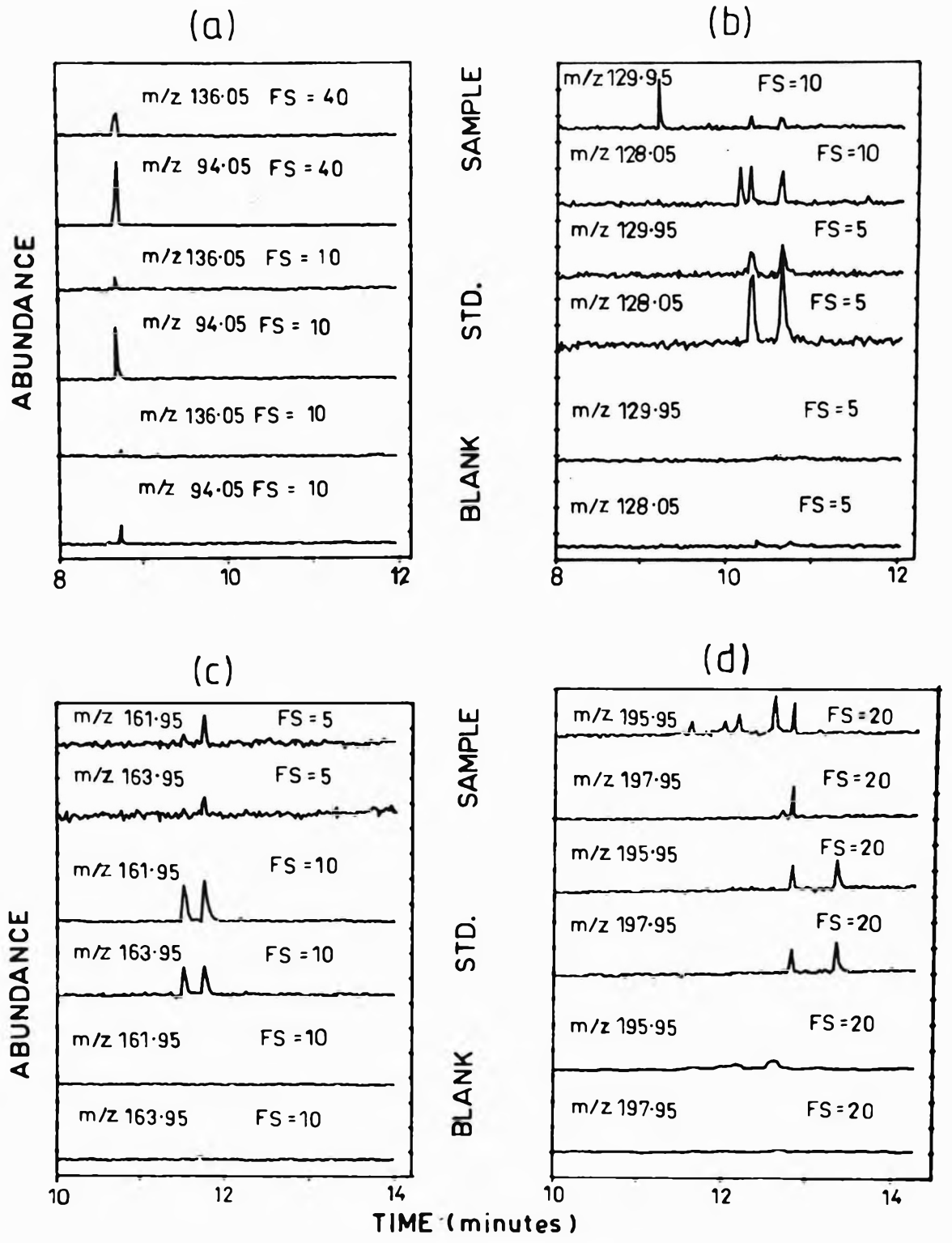

Figure 4. Analysis of water samples collected in February 1985 at potable water treatment plants. Typical SIM replots for acetylated extracts of city 6 and standards (400 pg). (a) phenol, (b) monochlorophenols, (c) dichlorophenols, (d) trichlorophenols.

essentially similar recoveries except for tetrachlorophenol, trichloroguaiacol, pentachlorophenol, and tribromophenol (Table 4).

The levels of halogenated phenols (corrected for blanks) in water samples collected in February 1985 at water treatment plants in 6 Canadian cities are reported in Table 5 and typical SIM replots of the acetylated extracts are shown in Figures 4a to 4d for phenol, monochlorophenols, dichlorophenols, and trichlorophenols. For each sample the concentration of halogenated phenol was calculated for the 2 ions monitored; the values reported in Table 5 are the mean of both ions in duplicate samples. Results for the 2 ions and the duplicate samples were usually in good agreement with relative standard deviations generally better than 25%, although, as indicated in some cases, halogenated phenols were found in only one of the duplicate samples. Treated water samples showed a variety of halogenated phenols which were not usually present in the raw water, indicating that these compounds were formed in the treatment process. The formation of these compounds cannot be entirely attributed to reaction of chlo-

rine with phenol because halogenated phenols were found in the treated water when no significant levels of phenol were present in the corresponding raw water sample. Further studies on the chlorination/bromination mechanism are in progress.

In conclusion, the in situ acetylation procedure is superior to the pentafluorobenzylation procedure for the determination of phenols in water samples because of fewer interferences, ease of handling, better blanks, and higher recoveries obtained. Between GC-ECD and GC-MS, the latter is considered superior, despite the longer analysis time, because all the halogenated phenols can be detected and quantitated and there are few problems with background interferences.

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FEEDS

Rapid Method for Determination of 2-Hydroxy-4-(Methylthio)butanoic Acid in Poultry Feeds by Capillary Isotachophoresis

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Capillary isotachophoresis, which involves the separation of charged species under an electric field, has been applied to the rapid determination of 2-hydroxy-4-(methylthio)butanoic acid at 0.04–0.50% concentration levels in corn/soy-based poultry feeds, using conductivity detection. The procedure merely involves a 15 min cold water extraction of the sample and a 15 min analysis after injection of the filtrate into the instrument. Since only charged species migrate and non-ionic species stay virtually at the front of the column, extraordinary selectivity can be achieved. The isotachophoresis method is an order of magnitude faster than the gas chromatographic method reported recently and also provides information on HMB-monomer/dimer ratio in the same run. The sample recoveries exceeded 90% in all concentration ranges studied with coefficients of variation less than ± 10%.

In the past 5 years, liquid methionine sources such as Alimet® (Monsanto Co.) have been used in poultry rations more and more compared to dry methionine sources. Over 60% of the U.S. poultry industry is currently using a liquid methionine source to improve chick and turkey poult growth, to increase egg production in layer hens, and to enhance performance in breeder flocks. This change has increased the need for quantitative measurement of addition levels in these diets to maintain good quality control as formulators change from solid to liquid sources.

Day et al. (1) reported a preparative gas chromatographic (GC) method for the determination of 2-hydroxy-4-(methylthio)butanoic acid (HMB) in feeds, using tetradecane as an internal standard related to the disilyl derivative of HMB monomer. Feit et al. (2) of Hazleton Laboratories of America shortened the GC method by freeze-drying the filtrates to yield hygroscopic residues for silylation. Although precision and accuracy of the GC method are excellent, the preparative chemistry is tedious and time-consuming (about 12 h for single sample runs).

In this paper, we describe the development of a rapid and reliable method for the analysis of poultry feeds for HMB, the active ingredient in Alimet, using isotachophoresis (ITP). Unlike the GC method, the ITP analysis also provides the HMB/HMB-dimer distribution ratio in a single run. ITP involves the movement of only charged species in an electric field, resulting in extremely simple sample preparation with excellent selectivity.

Although the principles governing ITP separation are quite old, it was Martin and Everaerts (3) who first systematically explored the theoretical and practical aspects of the technique. The first capillary tube apparatus for analytical ITP was built by Everaerts and Verheggen (4). Recently, ITP has been widely used in the analysis for organic acids and bases, amino acids, nucleotides, peptides, proteins, metal ions, fatty acids, and pharmaceuticals (5, 6).

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Experimental

Apparatus

(a) *ITP unit.*—LKB Model 2127 (Bromma, Sweden) Tachophor Analyzer with high voltage power supply and conductivity detector.

(b) *Capillary tube.*—0.5 mm id × 220 mm long Teflon tubing with Omnifit end connectors.

(c) *Recorder.*—Kipp and Zonnen (Netherlands) Model BD 41 2-channel strip chart recorder.

(d) *Data handling/current and recorder control.*—Hewlett-Packard Model 85 personal computer with custom-built data and control system (7).

Reagents

Unless specifically stated, all chemicals used were obtained from Sigma Chemical Co., St. Louis, MO 63178.

(a) *Leading electrolyte.*—Mix 20 mL 0.1M HCl with 40 mL 0.5% hydroxypropylmethylcellulose (HPMC) (Aldrich Chemical Co., Milwaukee, WI 53233) and dilute to 200 mL with DI water. Adjust to pH 6 by adding small amounts of solid L-histidine. HPMC is added to leading electrolyte to improve viscosity and to minimize convection effects.

(b) *Terminating electrolyte.*—Dissolve 0.39 g 2-(*N*-morpholino)ethane sulfonic acid in 200 mL DI water. Adjust to pH 6 by addition of solid tris(hydroxymethyl)aminomethane.

(c) *2-Hydroxy-4-(methylthio)butanoic acid (HMB).—1000 ppm stock solution*—Accurately weigh 0.1000 g HMB under blanket of dry nitrogen and dissolve in 100 mL DI water in volumetric flask. (HMB having purity of 98%, mp 42°C, was synthesized in our laboratory. Small quantities of HMB standard are available on request and approval of Monsanto Co.) *HMB standard solution.*—Prepare 50, 100, 200, and 400 ppm HMB standard solutions by appropriate dilution of stock solution.

Store electrolytes and HMB standard solutions in refrigerator (ca 5°C).

Procedure

Electrolyte blank.—Flush capillary with fresh leading and terminating electrolyte solutions. Turn current to 200 micro amps (μa) and let separation take place for ca 6 min. Then turn separation current down to 50 μa and start recorder to commence detection. Use 2 channels of recorder to monitor both conductivity and differential conductivity signals. After detecting leading and terminating electrolyte ions (ca 9 min), turn current and recorder chart drive off. This represents electrolyte blank and indicates system performance. Alternatively, HP-85 computer system can be used for automatic current programming, recorder chart drive control, and termination of run (7).

Calibration.—Flush capillary with fresh electrolytes. Inject 5 μL 50 ppm HMB standard at interfacial region of leading and terminating electrolytes. Turn current on first to 200 μa and follow procedure described above. In a similar manner,

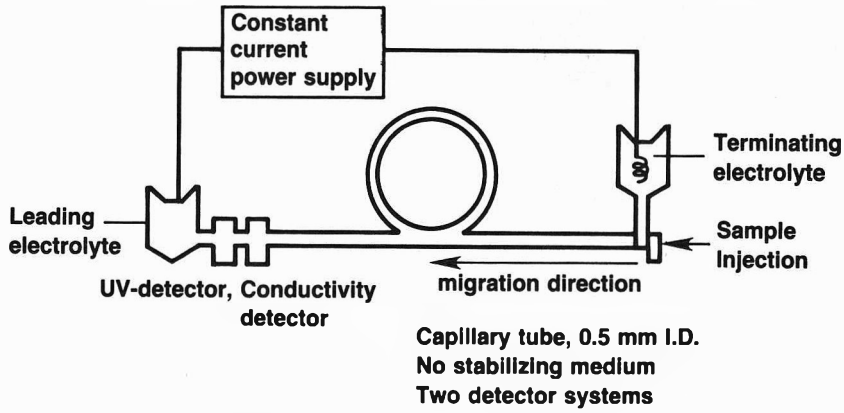

Figure 1. Analytical isotachophoresis.

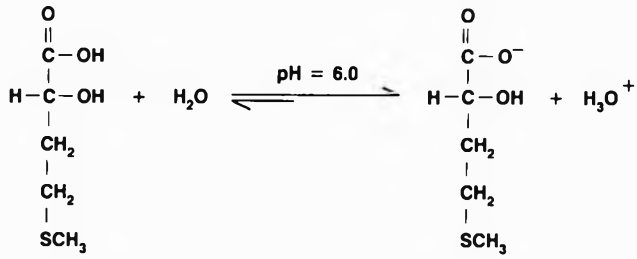

Figure 2. Dissociation of 2-hydroxy-4-(methylthio)butanoic acid.

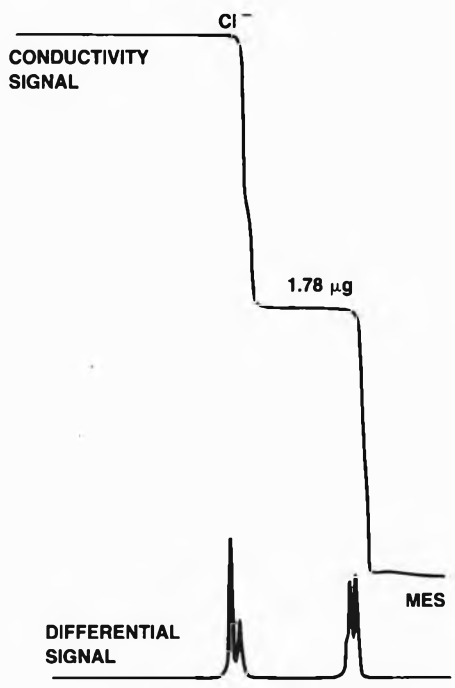

Figure 3. Isotachopherogram for HMB.

develop tachopherograms for 100, 200, and 400 HMB standards. These standards correspond to 0.25, 0.50, 1.00, and 2.00 μg HMB injected.

Sample Analysis

Grind corn/soy-based feed sample to powder consistency, using grinding mill. Accurately weigh ca 1 g sample in glass vial and extract with 10 mL DI water for 15 min on laboratory shaker. Filter (or centrifuge) sample through Gelman Acrodisc® filter, inject 5 μL filtrate into tachophor, and analyze as described above. Run blank in same manner using unsupplemented HMB corn/soy sample.

Calculation

Measure zone width corresponding to each HMB standard from differential conductivity signal in tachopherogram. Manual measurement with ruler or HP-85 computer system can be used for this purpose (7). Construct calibration curve by plotting zone width against concentration of HMB in μg. Interpolate sample zone width in calibration graph and read HMB concentration in μg.

$$\text{HMB in sample, g\%} = (\mu\text{g HMB in 5 } \mu\text{L sample} \times 2000 \times 100)/(10^6 \times \text{g sample})$$

Discussion

Isotachophoresis is the selective separation of ions at the interface of a leading and a terminating electrolyte under the influence of an applied electric field. The choice of the electrolyte system is dictated mainly by the ionization constant of the ionic species to be measured. The main requirement of the technique is that the sample ions being separated should have electrophoretic mobility between the leading and the terminating electrolytes. A schematic diagram of an ITP unit is shown in Figure 1 to illustrate the basic components (8). ITP does not involve conventional columns or packing, or loss of column sensitivity. If for some technical reasons the ITP run has to be aborted, one can flush the contents of the capillary unit and recharge with fresh electrolytes. In ITP, the electrolyte support-medium where ionic separation takes place is a capillary Teflon tubing.

The sample is injected at the interface of the leading electrolyte and the terminating electrolyte. Conductivity or UV absorbance is used to detect the movement of the leading electrolyte, the sample ions, and the appearance of the terminating electrolyte which marks the end of the run. The dissociation constant of butanoic acid is 1.34×10^{-5}. HMB, which for the most part is functionally and structurally similar to butanoic acid, has a *pKa* greater than butanoic acid. With these facts in mind, we evaluated the specific pH range of 5.0 to 7.0 with the selected electrolytes to achieve optimum ionization of HMB. The dissociation of HMB at pH 6.0, as listed in Figure 2, allows the separation and conductivity detection of HMB by using aqueous histidine hydrochloride as the leading electrolyte and MES (2-(*N*-morpholino)ethane sulfonic) acid as the terminator.

The isotachopherogram for HMB standard is illustrated in Figure 3. The quantitation of HMB from this distinct and sharp isotachopherogram agrees well with the GC assay for

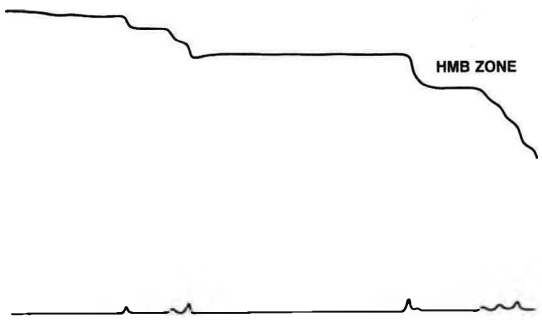

Figure 4. Isotachopherogram for extracted basal feed.

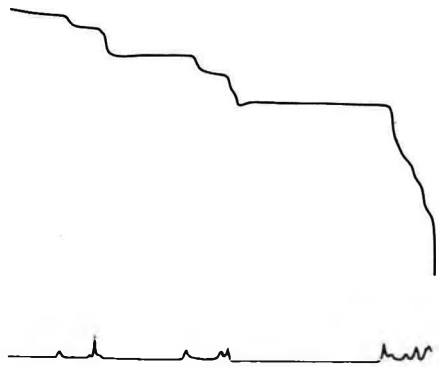

Figure 5. Isotachopherogram for extracted feed sample.

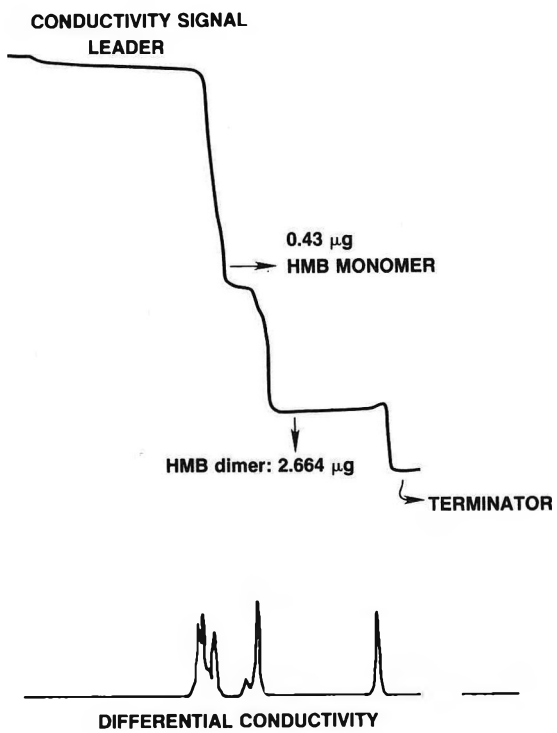

Figure 6. ITP profiles for HMB monomer and dimer.

HMB which is greater than 98%. A plot of the concentration of HMB in μg vs zone width in mm yields a calibration graph with excellent linear relationship (R^2 = 0.99). The sample preparation we used to separate HMB from feeds is much simpler than Day's method or Feit's modification. This method requires only 30 min for each run, 15 min for sample preparation, and 15 min for ITP measurement.

Table 1. Recovery and precision of ITP method for HMB-supplemented corn/soy test feed[a]

Theory, %	Mean recd, %	% Recd	SD	CV, %
0.048	0.050	103.8	0.0045	9.1
0.126	0.115	91.1	0.0044	3.9
0.196	0.199	101.7	0.0109	5.5
0.402	0.392	97.4	0.0108	2.8

[a]Based on 5 replicate analyses at each level of supplementation.

The isotachopherogram for the basal feed or the control feed (Figure 4) shows no HMB or background in the HMB region of measurement. On the other hand, an extraction of a supplemented feed (Figure 5) shows HMB in the designated region. By using this approach and spiking studies on common feed ingredients, analytical precision and accuracy are ensured.

In the ITP method, HMB is related to an HMB standard having purity greater than 98%. HMB dimer synthesized in our laboratories was greater than 95% pure. Since Alimet contains about 18–20% dimer and a small amount of higher oligomers, the isotachopherogram for HMB "prep" dimer was developed using the previously mentioned electrolytes. The isotachopherogram for this "prep" dimer was clean and distinct, and the assay agreed with the GC assay of 95% (Figure 6). The zone width of the HMB dimer plateau could be accurately measured by the differential signal and a calibration curve developed for HMB dimer showed a linear relationship (R^2 = 0.99). With pure HMB, pure HMB "prep" dimer, developed isotachopherograms for these materials, and calibration curves, we could use a rapid, cold water extraction technique to measure the level of HMB supplementation. The precision and accuracy for this ITP method is listed in Table 1. The dimer concentration in stored feed supplements having less than 0.4% HMB was found to be negligible.

To check the ITP method against the GC method, 70 commercial feed samples were analyzed by both methods. The results of these analyses showed exceptionally good agreement between methods. For the purpose of comparison, the data were analyzed by rank analysis using SAS, model fitted, and showed that a linear relationship does exist between the 2 methods with a high degree of accuracy. The correlation coefficient between the percent HMB in the feed samples from the 2 methods was 0.96 for 65 feed samples in the range of 0.01–20.0% HMB. These data are plotted in Figure 7 for the typical range of supplementation in poultry rations, 0.05–0.25% for HMB. The lack of sensitivity of the GC method at very low levels of HMB does not allow good correlation between methods below 0.05%.

In the HMB region of measurement, no interferences were detected from vitamins, fats, and minerals that are commonly added to poultry rations to complete nutritional balance. Therefore, the described experimental conditions are quite selective for the analysis of HMB in typical broiler rations.

Preliminary experiments for the analysis of HMB in silage samples suggested that further method development is necessary to achieve better correlation between the ITP and the GC methods. The silage matrix appears markedly different than the corn/soy matrices with respect to interferences in the HMB region of silage isotachopherograms.

Acknowledgments

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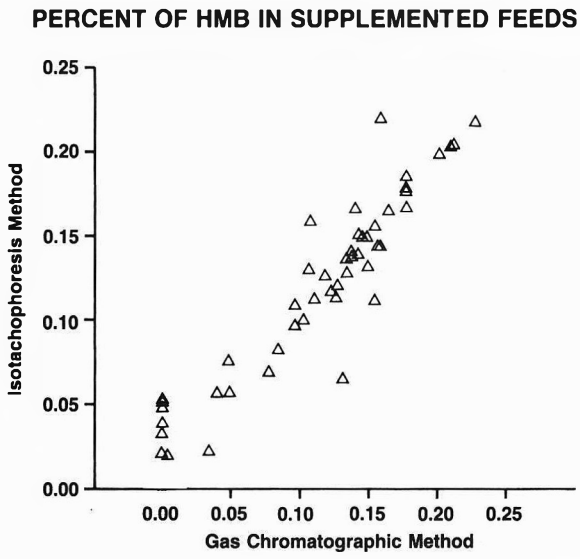

Figure 7. Statistical comparison of ITP and GC methods of analysis for HMB.

lytical assistance of R. R. Flynn and I. R. Putnam. We also thank R. L. Hintz for statistical evaluation of the data listed herein and the preparation of the manuscript by N. L. Litzsinger.

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COLOR ADDITIVES

Liquid Chromatographic Determination of Leuco Base in FD&C Blue No. 1

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Methods are described for the determination of leuco base in FD&C Blue No. 1 by reverse phase liquid chromatography and for the preparation and standardization of leuco base stock solution. The stock solution is prepared by reductive titration of the color with $TiCl_3$. Solutions of the color and of leuco base are chromatographed by isocratic elution, which is followed by a wash and equilibration that can be omitted for screening. Peak area and height calibrations were linear. At the specification level, the 99% prediction limits were 5.00 ± 0.14% (area) and 5.00 ± 0.37% (height). Limits of determination were 0.29% (area) and 0.73% (height) at the 99.5% confidence level. Recoveries were 97–101% for leuco base added to FD&C Blue No. 1 at levels of 1–6%.

FD&C Blue No. 1 (Colour Index No. 42090) is manufactured by oxidation of the leuco base with lead dioxide or sodium dichromate (Figure 1) (1). The color may be used in the United States in food, drugs, or cosmetics after the Food and Drug Administration (FDA) certifies that each lot of the color additive meets published specifications, including a limit of 5.0% leuco base calculated as the trisodium salt (2).

Leuco base was previously determined by FDA with a lengthy procedure involving air oxidation of a solution of the color and cuprous chloride and measurement of the increase in color absorbance (3). However, it was difficult to reproduce the resulting change of ≤ 5% in absorbance. A similar chloranil oxidation method (1) was also reported as difficult to reproduce (3).

A method for the determination of leuco base in FD&C Blue No. 1 by reverse phase liquid chromatography (LC) is reported here. A stock solution of leuco base is prepared by reductive titration of the color with $TiCl_3$ (Figure 1) (4). Solutions of FD&C Blue No. 1 and working solutions of leuco base are chromatographed. The area or height of the leuco base peak in each chromatogram is measured for standardization, calibration, and LC analysis. The concentration of leuco base in the stock solution is calculated from the titration and chromatography data. A calibration line is calculated by least squares linear regression from the areas or heights of the leuco base peak in chromatograms of the working solutions and a blank. The percentage of leuco base in a color sample is calculated from the calibration line and the area or height of the leuco base peak in the chromatogram of a solution of the color. Once the leuco base stock solution is prepared and standardized and the chromatography system is set up, individual samples can be analyzed in 20 min or screened in 10 min.

Experimental

Apparatus

(a) *$TiCl_3$ titrator.*—Described in ref. (4) for determining total color.

(b) *Liquid chromatograph.*—With gradient capability. (A system with eluant change capability may be used, but minor changes in eluant strength will be difficult with different columns or as the column ages.) A Waters system (Waters Associates, Milford, MA 01757) and Waters RCM C-18 column (System 1) were used for development of the method (standardization, calibration, recovery, and limited survey). To confirm that the method was robust and that other columns might be used, a Varian system (Varian Associates, Inc., Palo Alto, CA 94303) and Waters NOVA-PAK column (System 2) were used for the survey (standardization, calibration, and survey). Both systems were satisfactory; however, unacceptably high back pressure developed more quickly with the NOVA-PAK column.

(c) *LC column.—(System 1).*—Waters RCM C-18, 10 μm particle size, 100 × 8 mm id, in RCM 100 compression unit. *(System 2).*—Waters stainless steel NOVA-PAK C-18, 4 μm particle size, 150 × 3.9 mm id. Ambient temperature was used; however, temperature control is strongly recommended for quantitation based on peak height. Other C-18 columns were also used successfully.

(d) *Injector.—(System 1).*—Waters Model 710B WISP autoinjector set at 20 μL. *(System 2).*—Varian Model 8000 autosampler with 20 μL autoloop valve injector. Injection volume of 25 μL was also used.

(e) *Pumps and flow controller.—(System 1).*—Waters Model 720 system controller with 2 Waters 6000A pumps. Elution program beginning at injection at 2.0 mL/min: 57% eluant B (remainder eluant A) for 10.0 min, to 80% eluant B in 0.1 min, 80% eluant B (wash) for 2.9 min, return to 57% eluant B in 0.1 min, and equilibrate for 3.9 min. Run time is 10 min; equilibration delay is 7 min. The effective wash-equilibration delay is 9 min since automatic injection takes 2 min. *(System 2).*—Varian Vista Series 5000 system. Elution program beginning at injection at 1.2 mL/min: 55% eluant B for 9.9 min, to 80% eluant B in 0.1 min, 80% eluant B for 4 min, return to 55% eluant B in 0.1 min, and equilibrate for 11 min. For screening, the wash and equilibration may be omitted.

(f) *Detector.*—Waters Model 440 dual wavelength UV-visible detector set at 254 nm and 0.2 AUFS. Qualitative detection at 405, 546, or 625 nm aids in distinguishing the leuco base peak from color peaks.

(g) *Data system.—(System 1).*—Waters Model 730 data module. *(System 2).*—Varian Model 401 data system.

Reagents

(a) *Water for LC eluants.*—Distilled and passed through Milli-Q water purifier (Millipore Corp., Bedford, MA 01730).

(b) *Methanol.*—Omnisolv (EM Science, Gibbstown, NJ 08027).

(c) *Ammonium acetate.*—"Baker Analyzed" reagent crystals (J. T. Baker Chemical Co., Phillipsburg, NJ 08865).

(d) *Titanous chloride ($TiCl_3$).*—Stabilized 20% solution (Fisher Scientific Co., Pittsburgh, PA 15219).

(e) *Sodium hydrogen tartrate.*—Fisher certified.

Solutions

(a) *LC eluants.—(System 1).—Eluant A.* Aqueous 0.1M ammonium acetate. Dissolve 7.708 g ammonium acetate in

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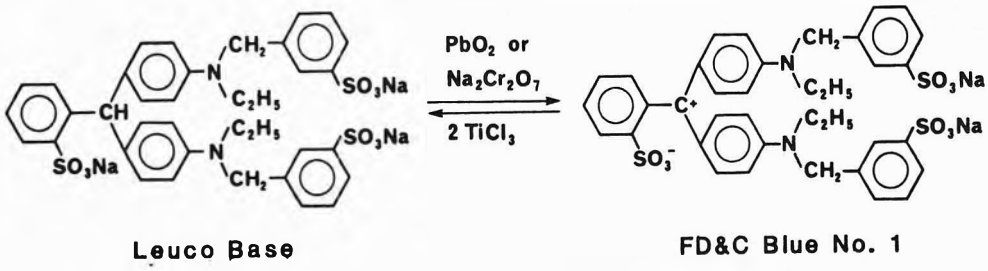

Figure 1. Interconversion of leuco base and FD&C Blue No. 1 (*m,m'*-isomers shown).

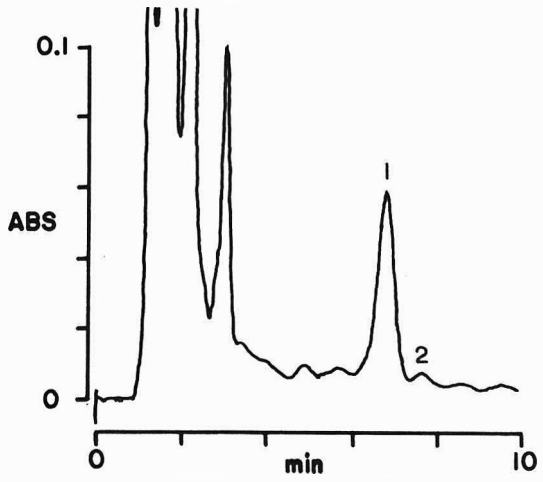

Figure 2. Chromatogram of FD&C Blue No. 1 (lot 5, Table 4). 1 = major isomers; 2 = minor isomer(s).

water and dilute to 1 L. *Eluant B.* Methanolic 0.1M ammonium acetate. Dissolve 7.708 g ammonium acetate in methanol and dilute to 1 L. *(System 2).—Eluant A.* Aqueous 0.2M ammonium acetate. Dissolve 15.417 g ammonium acetate in water and dilute to 1 L. *Eluant B.* Methanol.

(b) *FD&C Blue No. 1 solution.*—5 g/L. Accurately weigh 0.5 ± 0.005 g color. Transfer to 100 mL volumetric flask with ca 75 mL water and vigorously swirl contents of flask. After 15–30 min of intermittent swirling, dilute contents of flask to volume if no solid has settled.

(c) *$TiCl_3$ standard solution.*—0.1N; previously described (4).

(d) *Leuco base working standard solutions.*—Dilute 5, 10, 15, 20, 25, 30, and 35 mL aliquots of one stock solution to 100 mL. Prepare confirmatory working solution of leuco base by diluting 10–30 mL aliquot of second stock solution. These working solutions contain the equivalent of ca 1–7% leuco base in 0.5 g FD&C Blue No. 1/100 mL.

Preparation of Leuco Base Stock Solution

Certified FD&C Blue No. 1 with a low concentration of leuco base is preferred, but even the color to be analyzed may be used. In duplicate, accurately weigh 0.5 ± 0.005 g FD&C Blue No. 1 and transfer to 500 mL wide-mouth Erlenmeyer flask. To each flask, add 21–22 g sodium hydrogen tartrate, 250–300 mL water, and 2 or 3 silicone carbide boiling chips. Heat to boiling and titrate with $TiCl_3$ standard solution. There is no difficulty if a few extra drops of titrant are added to test the endpoint, as long as the true endpoint is recorded. Wash off titration apparatus into Erlenmeyer flask and quantitatively decant solution into 500 mL volumetric flask. Let solution cool and dilute to volume. The standardization of the stock solution is described below. In our laboratory a stock solution was stable for 1.5 yr when stored in the dark, but it required filtering to remove mold.

The FD&C Blue No. 1 used in the titration must also be analyzed for leuco base by LC.

Liquid Chromatography

Sparge eluants with helium, if desired, and set up gradient and detector. Prime and purge pumps and injection system. Wash column with water, then 100% eluant B for 10 or 20 min, and then initial eluant for 7 or 11 min at 2.0 or 1.2 mL/min for System 1 or 2, respectively. Chromatograph 2 preliminary water blanks.

For combined standardization and calibration, chromatograph, in random order, 7 working solutions, confirmatory working solution, at least 1 water blank, and 0.5% solution prepared from FD&C Blue No. 1 used for titration. As described below, calculate concentration of leuco base in stock solution.

For calibration using previously standardized leuco base stock solution, chromatograph, in random order, working standard solutions and at least 1 water blank.

For analysis, chromatograph FD&C Blue No. 1 solution(s) as shown in Figure 2 and water blank. If analysis is not concurrent with calibration, prepare and chromatograph working standard solution at specification level or other level of interest. The data from the chromatography of this standard solution should be within the prediction interval of the calibration line.

If this method was not used previously, chromatograph the most concentrated working solution of leuco base and 0.5% solution of FD&C Blue No. 1. Confirm that leuco base peak is well separated from color peaks and that leuco base elutes within reasonable time (k' = 3–5). If necessary, increase or decrease % eluant B to decrease or increase retention time. (An increase of 2% eluant B produced a decrease of 1.5 min in retention time in System 1). Prepare and chromatograph 0.01 dilution of stock solution. Adjust detector attenuation for proper display at level of interest and adjust integration parameters for proper quantitation, especially at lowest level.

With System 1, chromatographic peaks attributed to isomers of the color were followed by a peak with a tailing shoulder attributed to isomers of leuco base. With System 2, 2 peaks are attributed to the isomers of the leuco base as shown in Figure 2. Use the total area of the 2 peaks or the height of the major peak in quantitation. Error due to the difference in the absorptivities of the isomers is assumed to be negligible, and this error may be eliminated by using the color to be analyzed to prepare the leuco base stock solution.

Calculations

Titration.—Calculate percent color in FD&C Blue No. 1 used for titration as follows:

$$\% \text{ Color} = (V_t \times N_t \times 39.65)/W_t$$

Table 1. Standardization and calibration data[a]

Diln	Without FD&C Blue No. 1		With FD&C Blue No. 1		Without FD&C Blue No. 1		With FD&C Blue No. 1		Added leuco base,[c] rel. %
	t_R,[b] min	Peak area, kct	t_R,[b] min	Peak area, kct	t_R,[b] min	Peak ht, kct	t_R,[b] min	Peak ht, kct	
0	—(6)	0	7.20(8)	395.96	—(6)	0	6.52(8)	1.215	0
0.0050	6.95(8)	449.25	7.13(3)	952.63	6.60(8)	1.182	6.52(3)	2.510	0.0971
0.0100	6.86(1)	907.41	7.22(10)	1380.32	6.90(1)	2.339	6.56(10)	3.677	0.1942
0.0200	6.86(2)	1760.97	7.18(5)	2250.59	6.84(2)	4.648	6.46(5)	5.918	0.3883
0.0500	6.81(5)	4445.16	7.09(1)	4911.28	6.71(5)	11.792	6.49(1)	13.133	0.9708
0.100	7.03(10)	9032.24	7.24(7)	9305.40	6.53(10)	24.377	6.46(7)	24.805	1.942
0.150	6.81(3)	13244.7	7.26(9)	13934.2	6.79(3)	34.961	6.48(9)	39.155	2.913
0.200	6.86(7)	18056.2	7.12(2)	18413.9	6.62(7)	47.751	6.49(2)	48.039	3.883
0.250	7.03(11)	22303.8	7.16(4)	22284.6	6.52(11)	59.218	6.45(4)	61.504	4.854
0.300	6.81(4)	26520.8	7.24(11)	27430.6	6.76(4)	67.191	6.53(11)	74.454	5.825
0.350	6.98(9)	31695.1	7.17(6)	32079.1	6.56(9)	83.549	6.40(6)	90.695	6.796
Confirmatory working solution:									
0.100	6.84	9083	—	—	6.81	23.187	—	—	—
FD&C Blue No. 1 used for titration (0.5024 g/100 mL):									
—	6.86	378.0	—	—	6.65	1.308	—	—	—

[a]System 1 used. Abbreviations: t_R = retention time of leuco base peak; kct = 1000 integrator area or height counts.
[b]Number in parentheses indicates order of analysis.
[c]Relative to 0.5 g FD&C Blue No. 1/100 mL; $[leuco]_{stock}$ = 0.9708 g/L.

Table 2. Least squares linear regression analysis of standardization data for leuco base in FD&C Blue No. 1[a]

Parameter	Peak area		Peak height	
	Without FD&C Blue No. 1	With FD&C Blue No. 1	Without FD&C Blue No. 1	With FD&C Blue No. 1
Dilution data:				
Slope ± conf. int., kct/% leuco[b]	89657 ± 1497		233.79 ± 10.04	
Intercept ± conf. int., kct[b]	−22.02 ± 267.24		0.1378 ± 1.793	
Corr. coeff.	0.99988		0.99922	
Rel. % leuco data:				
Slope ± conf. int., kct/% leuco[b]	4617.7 ± 77.1	4639.8 ± 31.9	12.041 ± 0.517	12.807 ± 0.615
Intercept ± conf. int., kct[b]	−22.02 ± 267.25	420.59 ± 110.08	0.1378 ± 1.793	0.75262 ± 2.132
Corr. coeff.	0.99988	0.99998	0.99922	0.99902
Upper limit of blank, kct[c]	639.4	694.7	4.575	6.029
c_L, % leuco[c]	0.29	0.12	0.73	0.81
Meas. corres. to c_L, kct	1294	968	8.915	1.118
Prediction limits, % leuco[b]	5.00 ± 0.14	—	5.00 ± 0.37	—

[a]System 1 used. Abbreviations: conf. int. = confidence interval; kct = 1000 integrator area or height counts; c_L = limit of determination.
[b]Confidence level 99%.
[c]Confidence level 99.5%.

where V_t = volume (mL) of titrant consumed, N_t = normality of titrant, W_t = weight (g) of color titrated, and 39.65 ((396.5 g FD&C Blue No. 1/equivalent) × (0.001 L/mL) × 100) = conversion factor. If the average deviation of the percent color for the duplicate titrations is >1%, the titrations must be repeated.

Standardization.—Peak height may be used in place of peak area. The standards contain leuco base that was originally in the color plus leuco base from reduction of the color. From chromatograms of working solutions, plot peak area vs dilution of stock solution (e.g., 0.35) used to prepare each working solution.

$$\text{Area} = (\text{slope}_d \times \text{dilution}) + \text{intercept}$$

Calculate slope_d and intercept of this standardization line by least squares linear regression. The intercept should not be distinguishable from zero. If the data from the chromatogram of the confirmatory working solution are within the prediction interval of the standardization line, the second stock solution may be discarded. If the data are not within the prediction interval, the titrations must be repeated.

From titration data, slope and intercept of standardization line, and chromatogram of solution prepared from FD&C Blue No. 1 used in titration, calculate slope of calibration line for peak area vs relative % leuco base in working solutions (relative to 0.5 g FD&C Blue No. 1/0.1 L) from the following equation (derived in Appendix):

$$\text{Slope}_{\%} = [(5\text{ g/L})/(V_t \times N_t \times 40.84)] \times [(\text{slope}_d \times V_{stock}) - ((\text{area}_{b'} - \text{intercept}) \times W_t \times V_{b'}/W_{b'})]$$

where V_{stock} = volume (L) of leuco base stock solution (usually 0.5 L); $W_{b'}$ = weight (g) of color, $V_{b'}$ = volume (L) of color solution (e.g., 0.5024 g in 0.1 L), and $\text{area}_{b'}$ = peak area from chromatogram of solution prepared specifically (′) from FD&C Blue No. 1 used for titration; and (5 g/L) and 40.84 ((408.4 g leuco base trisodium salt/equivalent) × (0.001 L/mL) × 100) = conversion factors.

Calculate % leuco base in FD&C Blue No. 1 used for titration as follows:

$$\%\ \text{Leuco}_{b'} = [(\text{area}_{b'} - \text{intercept})/\text{slope}_{\%}] \times (5\text{ g/L})/(W_{b'}/V_{b'})$$

This calibration equation (without primes) is used to calculate % leuco base in any FD&C Blue No. 1.

Calculate concentration (g/L) of stock solution, $[leuco]_{stock}$, as follows:

$$[\text{Leuco}]_{stock} = [(V_t \times N_t \times 0.4084) + (0.01 \times \%\ \text{leuco}_{b'} \times W_t)]/V_{stock}$$

Table 3. Recovery data for LC determination of leuco base in FD&C Blue No. 1[a]

	Peak area integration			Peak height integration		
Added, %	Found ± SD, %	Rec.,[b] %	CV, %	Found ± SD, %	Rec.,[b] %	CV, %
0	0.0878 ± 0.0068	—	—	0.1024 ± 0.0080	—	—
0.0971	0.2054 ± 0.0028	121	1.4	0.2019 ± 0.0042	102	2.1
0.971	1.029 ± 0.012	97	1.2	1.079 ± 0.035	101	3.2
2.912	2.932 ± 0.023	98	0.8	3.037 ± 0.058	101	1.9
5.825	5.752 ± 0.030	97	0.5	5.941 ± 0.052	100	0.9

[a]System 1 used. Quintuplicate analyses.
[b]Corrected for leuco base found in unfortified FD&C Blue No. 1.

Calibration.—A calibration line is determined for statistical analysis of the calibration data and for recalibration using a previously standardized stock solution. Calculate relative % leuco base in working solutions as follows:

$$\text{Rel. \% leuco} = 100 \times [\text{leuco}]_{\text{stock}} \times \text{dilution}/(5\ \text{g/L})$$

From chromatograms of working solutions, plot peak area vs relative % leuco base, and calculate by least squares linear regression the slope and intercept of the calibration equation as follows:

$$\text{Area} = (\text{rel. \% leuco} \times \text{slope}_{\%}) + \text{intercept}$$

If analysis is not concurrent with calibration and single point calibration is used, use calibration equation shown above to calculate expected peak area from chromatography of single working solution. Confirm that actual area is within prediction interval of calculated area. If area is not within prediction interval, recalibration may be needed. The retention time may have changed, especially if peak height quantitation is used, or an error may have been made.

Results and Discussion

Titration

A large volume of leuco base was needed. Three portions of FD&C Blue No. 1 of 0.5001, 0.5013, and 0.5004 g required 9.39, 9.40, and 9.42 mL 0.1264N $TiCl_3$, respectively. The color content was determined to be 94.1 ± 0.2% with excellent precision. The first 2 titrated solutions and washings were combined and diluted to 1 L for the first stock solution; the third was diluted to 500 mL for the second stock solution.

Standardization

Ten working solutions were prepared from the first stock solution and one working solution from the second as shown in Table 1. After 2 preliminary blanks were chromatographed, the working solutions, a blank, and a 0.5024 g/100 mL solution prepared from the FD&C Blue No. 1 that had been titrated were chromatographed twice for peak area and height quantitation (Table 1).

The plot of peak area (kct = 1000 integrator area or height counts) vs dilution was linear, with a slope_d of 89657 kct/dilution; the intercept did not differ from zero (Table 2). The data for the confirmatory working solution were within the prediction interval. The $\text{slope}_{\%}$ was 4617.5 kct/% and the intercept was −22.02 kct. The % leuco base in the FD&C Blue No. 1 used for titration was 0.086. The $[\text{leuco}]_{\text{stock}}$ was 0.9708 g/L.

The plot of peak height vs dilution was linear, with a slope_d of 233.79 kct/dilution; the intercept did not differ from zero (Table 2). The data for the confirmatory working solution were within the prediction interval. The $\text{slope}_{\%}$ was 12.040 kct/%, and the intercept was 0.1378 kct. The % leuco base in the FD&C Blue No. 1 used for titration was 0.097. The $[\text{leuco}]_{\text{stock}}$ = 0.9709 g/L. These values are in excellent agreement with those from area standardization.

Calibration

The relative % leuco base in each working solution was calculated (Table 1). Peak areas were plotted vs relative % leuco base, and the data were analyzed by least squares linear regression (Table 2) (5). The plot of the data was linear with a $\text{slope}_{\%}$ of 4617.7 kct/%, and the intercept was −22.02 kct as before. The plot of peak height vs relative % leuco base was linear with a $\text{slope}_{\%}$ of 12.041 kct/%, and the intercept was 0.1378 kct as before. The intercepts did not differ from zero. Limits of determination were 0.29 and 0.73% leuco base for peak area and height, respectively. Prediction limits at the specification level were 5.00 ± 0.14% (area) and 5.00 ± 0.37% (height). The greater variability in the peak height calibration is attributed in part to a trend of decreasing retention times (and presumably increasing peak heights) with increasing run number. Control of column temperature may improve the precision of peak height integration. The retention times in Table 1 ranged from 7.26 to 6.45 min over 2 days.

Peak area and height calibration plots for leuco base in the presence of FD&C Blue No. 1 (Table 1) were also linear. The peak area intercept differed from zero but did not differ from the % leuco base in the unfortified FD&C Blue No. 1 from the recovery studies (Table 3). The peak area slope did not differ from that previously found. The peak height intercept did not differ from zero nor from the % leuco base in the unfortified FD&C Blue No. 1. The peak height slope was significantly greater than before; the difference was attributed to shorter retention times.

Recovery Studies

Recovery solutions of FD&C Blue No. 1 were prepared in quintuplicate without added leuco base and with leuco base added at 4 levels. These solutions were chromatographed twice (area and height) in random order in sets of the different levels (Table 3). Chromatograms of water blanks contained no interfering peaks. Excellent precision and recoveries were demonstrated, with the exception of one excessive recovery based on peak area at the 0.0971% fortification level. This high value is perplexing and unexplained, since the precision and the recovery based on peak height were excellent. From the standard deviations of the determinations of unfortified FD&C Blue No. 1, the limits of determination were 0.03–0.04%. These values were calculated from

$$c_L = 4.604 \times \text{SD}_{\text{blank}}/\text{slope}_{\%}$$

in which a 99.5% one-sided *t*-value is used instead of $k = 3$ (6). These limits of determination suggest that the calibration limits of determination of 0.29% (area) and 0.73% (height) can be improved. More recent determinations have had calibration limits of determination of 0.1% (area).

Table 4. Duplicate analyses of certified FD&C Blue No. 1 by LC and oxidation methods

	Leuco base, %			
Lot	LC[a]		Oxidation	
1	0.19	0.29	3.31	1.30
2	0.17	0.28	3.03	1.43
3	0.21	0.33	1.15	1.28
4	0.09	0.15	<1.0	<1.0
5	0.79	0.78	1.7	2.8
6	0.28	0.37	2.1	2.1
7	0.31	0.31	1.8	1.7
8	0.77	0.86	1.7	1.4
9	0.09	0.11	<1.0	0
10	0.10	0.16	1.2	0
11	0.08	0.14	0	0.5

[a]System 2 used. Based on peak area integration.

Survey

Samples from 11 certified lots of FD&C Blue No. 1 had been analyzed and reanalyzed later by the oxidation method (3) with the lot identities unknown to the analyst in the second analysis (Table 4). Samples from the same lots were analyzed and then reanalyzed by the LC method with the lot identities unknown to the analyst in both analyses. Agreement between methods was very poor. Within-method precision by the LC method was excellent, whereas within-method precision by the oxidation method was poor. We believe that the difficulty in determining a small difference between 2 large absorbances accounts for the poor precision of the oxidation method. In the preparation of the 2 colored solutions compared, a 1% error in pipetting or dilution would produce a 50% error in the determination of 2% leuco base in FD&C Blue No. 1. Furthermore, 1 of the 2 blanks measured has an appreciable absorbance. The poor agreement between methods is, therefore, attributed to the imprecision of the oxidation method.

Conclusion

This LC method is a significant improvement over the previously used oxidation method since the LC method is amenable to automation and is more precise and much faster. Work continues on extending this method to the determination of intermediates and subsidiary colors in FD&C Blue No. 1 and to the analysis of FD&C Green No. 3.

Appendix

For plots of peak area vs concentration of working solution, where the concentration is expressed either as dilution of the stock solution or as relative % leuco base compared to 0.5 g FD&C Blue No. 1/0.1 L, the area data are the same and the calculated intercepts of the linear regression equations are the same. The linear equations

$$\text{Area} - \text{intercept} = \text{slope}_d \times \text{dilution}$$

$$\text{Area} - \text{intercept} = \text{slope}_{\%} \times \text{rel. \% leuco}$$

were set equal.

$$\text{Slope}_{\%} = \text{slope}_d \times \text{dilution/rel. \% leuco}$$

Into this equation, the following equations were substituted in turn:

$$\text{Rel. \% leuco} = 100 \times [\text{leuco}]_{stock} \times \text{dilution}/(5\ \text{g/L})$$

$$[\text{Leuco}]_{stock} = [(V_t \times N_t \times 0.4084) + (0.01 \times \%\ \text{leuco}_{b'} \times W_t)]/V_{stock}$$

$$\%\ \text{Leuco}_{b'} = [(\text{area}_{b'} - \text{intercept})/\text{slope}_{\%}] \times (5\ \text{g/L})/(W_{b'}/V_{b'})$$

Solving for $\text{slope}_{\%}$ yields the following equation:

$$\text{Slope}_{\%} = [(5\ \text{g/L})/(V_t \times N_t \times 40.84)] \times [(\text{slope}_d \times V_{stock}) - ((\text{area}_{b'} - \text{intercept}) \times W_t \times V_{b'}/W_{b'})]$$

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MEAT AND MEAT PRODUCTS

Development of Poultry Rapid Overnight Field Identification Test (PROFIT)

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A poultry rapid overnight field identification test (PROFIT) has been developed as a screening test which is practical, economical, and easy to perform and interpret for use in field environments to determine the presence of poultry tissue (chicken and turkey) in raw whole tissue or ground/formulated meat products. The basis of the test is an agar-gel immunodiffusion technique used with a printed template pattern and stabilized reagent paper discs. The test shows adequate sensitivity and specificity for its intended purpose. Key components are stable for at least 1 year if they are stored at refrigerator conditions. The design of the test is such that it can be made commercially available as a complete, stable, test kit suitable for use by any type of inspection program concerned with verification of poultry species in meat and/or poultry products that are subject to regulatory or quality controls.

Standards of identity and/or composition for livestock and poultry food products exist as specific regulations cited in Title 9 of the *Code of Federal Regulations* (1) as a means to assure unadulterated and accurately labeled meat and poultry products to the consumer. One important avenue available to assist in obtaining compliance with these regulations concerns the use of laboratory analysis of meat products to identify the species of animal tissue used in the product to assure that no adulteration or fraudulent substitution has taken place.

Animal tissue species have been successfully identified in the past by such techniques as the interfacial ring precipitin test (2), agar-gel immunodiffusion (3), enzyme-linked immunosorbent assay (4), radioimmunoassay (5), polyacrylamide gel electrophoresis (6), and thin layer isoelectric focusing in agarose (7) or polyacrylamide gel (8). Although all of those procedures have their own individual merits, they suffer from common problems of a relatively high cost per sample analysis (including shipping and results reporting), the need for a certain level of technical expertise on the part of the analysts, the use of special equipment or expensive and labile biochemical reagents, and most notably the need for performance in a formal laboratory environment. These disadvantages may become major obstacles to significant testing programs in federal, public, or private commercial institutions needing to analyze large numbers of samples.

Poultry (chicken and turkey) tissue represents a major source of protein, generally less expensive than red meat, which is consumed and imported throughout the world. These factors together with the regulated, increasing use of mechanically separated poultry produces a significant potential for the adulteration or substitution of red meat products by poultry. This can be supported by the observation that poultry represents one of the more commonly occurring species violations seen in the analysis of many samples in our laboratories during the past few years.

A need exists for a basic poultry screening system which is economical, easy to use and interpret, accurate, sensitive, capable of being produced in a stabilized, commercial kit form, and most important, capable of field applications such as an abattoir, import inspection station, or commercial meat product establishment. We studied the applications of principles of the very successful overnight rapid bovine identification test (ORBIT) (9), now commercially available, as a means to satisfy this need. In this report we present a basic poultry screening test, referred to as the poultry rapid overnight field identification test (PROFIT), which satisfies all of the previously stated objectives and can be used on any raw, whole meat tissue or ground/formulated meat (poultry) product.

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METHOD

Apparatus

(a) *Petri dish.*—Falcon No. 1006, tight lid, 50 × 9 mm (Falcon, Division of Becton, Dickinson and Co., 1915 Williams Dr, Oxnard, CA 93030), custom silk-screen printed (Granite Diagnostics Inc., PO Box 908, Burlington, NC 27215) with 4 lettered circles in pattern (Figure 1A) on outside of bottom plate.

(b) *Filtering cloth.*—Miracloth (Calbiochem-Behring, 10933 N Torrey Pines Rd, La Jolla, CA 92037).

(c) *Paper discs.*—BBL No. 31039 blank filter paper sensitivity test discs (Becton, Dickinson and Co., PO Box 243, Cockeysville, MD 21030).

(d) *Lyophilizer.*—Freezemobile II lyophilizer with Model 10 MR-SA single-shelf, vacuum drying chamber (The VirTis Co., Inc., Rt 208, Gardiner, NY 12525).

(e) *Bacteriological filter.*—Millipore Millex-GS, 0.22 μm filter unit (Millipore Corp., 80 Ashby Rd, Bedford, MA 01730).

(f) *Plate reader.*—Hyperion viewer with magnifier, Model No. 4040-100A (Hyperion, Inc., Miami, FL).

(g) *Dispo automatic analyzer beakers.*—Conical bottom, 4 × 0.5 mL, No. B-2713-35 (American Scientific Products, 1430 Waukegan Rd, McGaw Park, IL 60085), custom silk-screen printed (Granite Diagnostics Inc.) with 2 permanent, water-insoluble, measurement lines (Figure 1B) on the outside.

(h) *Wooden applicator sticks.*—6 in. long, No. A-5000-1 (American Scientific Products).

Reagents

(a) *Agar.*—Purified, No. 0560-01 (Difco Laboratories, PO Box 1058, Detroit, MI 48232).

(b) *Phosphate-buffered saline.*—0.85% NaCl solution containing 1.25 mL/L stock 0.25M KH_2PO_4 solution previously adjusted to pH 7.2 with 1N NaOH.

(d) *Merthiolate solution.*—Thimersal, NF powder (Elanco-Eli Lilly Co., Indianapolis, IN 46206), stock 1% aqueous.

(e) *Adjuvant.*—Freund's complete and incomplete adjuvants, No. 0638-60 and 0639-60 (Difco).

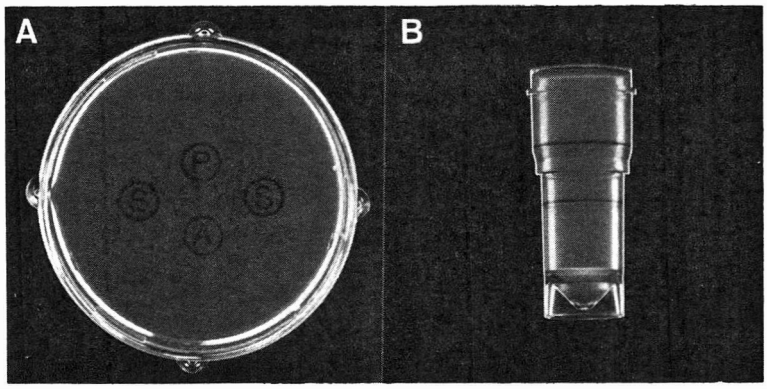

Figure 1. Important components of PROFIT: A, Agar-gel immunodiffusion plate with printed pattern for standardized disc placement. Lettered circles are 6.5 mm in diameter and 5 mm equidistant with respect to adjacent inner edges. The 2 sample circles (S) are 13.5 mm directly opposite each other (inner edges). B, Beaker calibrated and marked for ground/formulated meat sample treatment. Lower line is 10 mm above level bottom of beaker and upper line is 9 mm above lower line.

(**f**) *Powdered albumins.*—Chicken serum albumin, Fraction V, No. A-3014, and turkey serum albumin, Fraction V, No. A-4650 (Sigma).

(**g**) *Chicken serum albumin solution.*—Chicken serum albumin (**f**), 0.1% in phosphate-buffered saline (**b**), 0.22 μm Millipore filter-sterilized.

Agar-Gel Immunodiffusion Plate Preparation

Fill Petri dishes (**a**) with 4 mL level, bubble-free immunodiffusion agar prepared in the following manner: Make 1% concentration of purified agar (**a**) in pH 7.2 phosphate-buffered saline (**b**) and heat until agar is totally solubilized and clear. Filter hot agar solution under vacuum through single layer of filtering cloth (**b**) in Büchner funnel on side-arm flask; autoclave at standard conditions. After sterilization, aseptically add sufficient quantity of dye solution (**c**) to liquid agar and mix to effect a final 1:120 000 concentration of dye. Cool agar solution to 60°C in waterbath, add sufficient quantity of merthiolate solution (**d**), and mix to effect final merthiolate concentration of 1:10 000. Dispense agar directly into plates or let harden in stock quantities and store at room temperature until remelted for future use. Keep prepared plates in sealed double plastic bags at 4°C until needed.

Anti-Poultry Serum Preparation

Prepare suitably reacting anti-poultry serum of proper specificity and strength for use by immunizing goats with mixture of 2.5 mg each of chicken serum albumin and turkey serum albumin (**f**) in Freund's complete (primary injection) or incomplete (all secondary injections) adjuvants (**e**). Give intramuscular injections (5 mg) at monthly intervals in total volume of 2 mL: 1 mL into each of the 2 rear, hind leg biceps femoris muscle of each animal. Make trial bleedings 10–14 days after each injection. Let blood clot at room temperature, and separate and clarify serum by centrifugation at 1500 × *g* for 20 min. Monitor quality of each lot of antiserum for its specificity and strength by preparing stabilized reagent paper discs (described below) and observing reactivity against whole and ground heterologous and homologous tissue fluids within designed parameters of PROFIT procedure (described below).

Preparation of Stabilized Reagent Paper Discs

Prepare stabilized poultry reference antigen discs by impregnating blank paper discs (**c**) with 40 μL 0.1% chicken serum albumin solution (**g**). Prepare stabilized anti-poultry antibody discs by impregnating additional blank paper discs (**c**) with 40 μL of suitably reacting goat anti-poultry serum. Let both sets of paper discs absorb their reagents, and freeze-dry (**d**) overnight as previously described (9).

PROFIT Procedure

Remove prepared PROFIT agar-gel immunodiffusion plates from refrigerator and let equilibrate to room temperature. The 4 lettered circles (S, S, P, and A) should be readily visible through the green colored agar. Using fine-pointed forceps, carefully place flat on agar surface of PROFIT plate one anti-poultry antibody disc such that A lettered circle of template is completely and evenly covered by disc when viewed directly from above. In an identical manner, place one poultry reference antigen disc over P lettered circle of same plate. Sample discs may be prepared from either thawed, raw, whole muscle tissue or ground/formulated meat product samples. If sample is whole muscle tissue, make vertical slice with sharp knife to create a single slit about 38 mm deep in area free of fat and connective tissue. Use forceps to place one blank sample paper disc halfway into depth of slit, flat against one side of tissue. Gently squeeze slit together so that both sides of sample disc are in contact with meat tissue. Let disc remain in this position 10–30 s to absorb tissue fluids and appear obviously wet. If sample is ground/formulated type, place ca 1 g sample, well packed, into measured beaker (**g**) such that beaker is filled level with bottom black measuring line. Add ca 1 mL cold tap water to fill beaker level to top black measuring line. With one end of clean wooden applicator stick (**h**), gently mix sample and water to uniform emulsion. Tilt beaker 45° and immerse clean blank sample disc in emulsion to depth necessary for complete saturation. Remove excess fluid and meat particles from sample disc by wiping it on the beaker rim. Place sample disc, from either type of sample, as previously described, over one of the S lettered circles of PROFIT plate containing positioned reference antigen and antibody discs. Treat second test sample in identical fashion and place that sample disc over remaining unoccupied S lettered circle of same plate. Tightly seal lid on plate and leave undisturbed overnight (15–24 h) at normal room temperature to let reagents diffuse through agar and react. Examine plates in white, indirect light against flat black background, Hyperion viewer (**f**), for formation of characteristic immunoprecipitin line in agar area among the 4 positioned paper discs; make interpretations as to whether or not the samples contain poultry tissue.

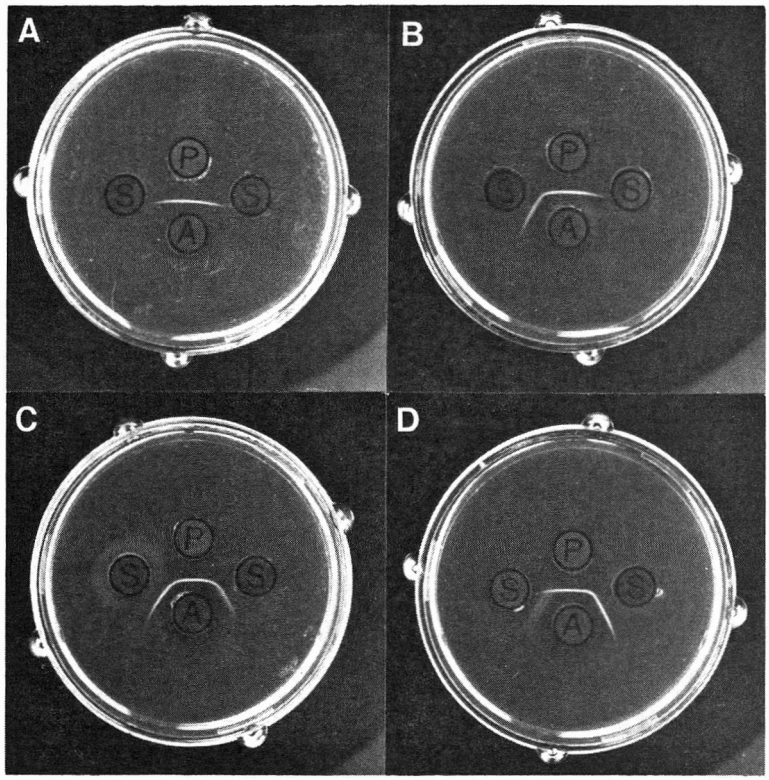

Figure 2. Typical PROFIT plate reactions resulting from routine sample analysis with goat anti-poultry antibody reagent discs: Plate A, both samples negative for poultry; plate B, sample on left positive for poultry; plate C, both samples positive for poultry; and plate D, only sample on right positive for poultry.

Optimum Reaction Parameters, Sensitivity, and Reagent Disc Stability

The reaction parameters of reagent disc distance and arrangement, depth of agar in the plate, reagent concentration, and incubation time and temperature were empirically studied as independent variables using homologous and heterologous tissue fluids on sample discs (described previously). Determination of these optimum conditions was necessary to assure specificity and visibility of the immunoprecipitin line in the PROFIT procedure.

The sensitivity of PROFIT as applied to ground meat mixture sample analysis was determined by testing composite samples prepared by mixing known percentages of poultry tissue, as adulterant, in ground red meat base tissue on a weight-to-weight basis. Three replicates of each composite sample were analyzed in this study. The presence of a visible sample immunoprecipitin line which fused completely with the poultry reference immunoprecipitin line was employed as the criterion for a positive detection reaction for a given percentage of poultry tissue.

Long-term stability of prepared PROFIT materials was assessed by subjecting the key component of reagent paper discs to a shelf stability study. Prepared anti-poultry antibody and poultry reference antigen paper discs were stored under different conditions (room temperature vs refrigerated at 4°C) and individual discs were removed periodically and tested for the quality of the resulting immunoprecipitin line on PROFIT agar plates.

Results

Performance Characteristics

Goat anti-poultry serum was commercially prepared (Granite Diagnostics Inc.) according to the procedure described. Suitable antiserum was generally available after the administration of 1–3 booster injections, which produced the desired intensity and specificity between reagent discs. The optimum reaction parameters of reagent disc distance and arrangement, agar depth in the plate, reagent concentration, and incubation time and temperature were found to be the same as those established for the ORBIT procedure (9). Determination of the reagent and sample discs distance and arrangement allowed for the production of a printed template pattern on the outside bottom of the plate to simplify standardized disc placement during analysis (Figure 1A).

Typical sample and reference reaction patterns resulting from these established test parameters are illustrated in Figure 2. Intense immunoprecipitin lines of identity (complete fusion) result from samples containing poultry proteins (plates B, C and D) with that of the poultry reference antigen. Occasionally a sample may produce nonspecific reaction lines (plate D, left sample) which make it easily distinguishable as a nonpoultry-containing sample due to the lack of identity (nonfusion) with the poultry reference antigen. Samples which contain smaller amounts of poultry proteins may produce immunoprecipitin lines of less intensity than those produced from samples containing a greater concentration of poultry protein (plate C, right sample compared to left sample); however, this does not cause any problems with correctly interpreting and identifying samples containing poultry.

A determination of the specificity of the goat anti-poultry serum in the proposed screening procedure tested against routine samples of several species of red meat and poultry, produced the following results: bovine (−), deer (−), horse (−), pig (−), sheep (−), red kangaroo *(Macropus rufus)* (−), chicken (+), turkey (+), duck (+), goose (+), pheasant (+), quail (+), partridge (+). All red meat species gave a negative reaction, while all poultry species gave a positive reaction, irrespective of the physical nature of the sample (ground or whole). The demonstration of reactivity of all poultry species

Table 1. Results of PROFIT laboratory and field trials on wide variety of blind screen (unknown) meat product samples

Product	Species composition[a]	Number of samples	Positive samples
Lab. trial:			
Meat balls with eggs & spices	bovine, chicken eggs	1	1
Ground beef	bovine, (chicken)	3	3
Ground beef	bovine, (turkey)	5	5
Ground beef	bovine, (turkey & pig)	1	1
Ground beef	bovine, (pig)	1	0
Beef patty mix	bovine, (pig)	1	0
Frank emulsion	bovine, pig, (chicken)	1	1
Veal	(turkey)	2	2
Ground pork	pig, (bovine)	1	0
Pork sausage patties	pig, (bovine)	2	0
Chicken breast	chicken	1	1
Boneless venison	deer	1	0
Total		20	14
Field trial:			
Ground beef	bovine	1	0
Frank emulsion	bovine, pig	2	0
Frank emulsion	bovine	3	0
Bologna emulsion	bovine	1	0
Bologna emulsion	bovine, pig	1	0
Boneless beef	bovine	1	0
Pork chop	pig	1	0
Pork sausage	pig	1	0
Frank emulsion	bovine, pig, chicken	2	2
Frank emulsion	chicken	20	20
Chicken breast	chicken	12	12
Bologna emulsion	chicken	1	1
Total		46	35

[a]Identity of species in all samples was confirmed by Ouchterlony agar-gel, 2-dimensional, double-immunodiffusion technique (3) using whole anti-species sera and tissue extracts of authentic reference tissue. Species given in parentheses represent known adulterant tissue present in the test samples used for lab. trial.

tested was as expected due to the designed nature of the screen test and the immunization protocol employed.

Sensitivity studies of the procedure determined that turkey could be detected at the 3% and 5% levels in pork and beef tissue bases, respectively, while chicken could be detected at the 10% and 7.5% levels in the same respective tissue bases (data not shown). These minimal detection levels were considered to be adequate in light of the design, intended application, and nature of the basic serological test procedure.

The long-term stability study of prepared PROFIT materials revealed that reagent discs stored in screw-cap vials in the refrigerator (4°C) were still capable of producing immunoprecipitin lines equal to those produced by freshly prepared reagent discs for a maximum stability period extending to at least 1 year (data not shown). Antibody discs stored at room temperature, however, showed a significant reduction in reactivity. This stability period for refrigerator-stored discs was considered to be highly adequate for kit production and use.

Performance Trials

To demonstrate the practicality, reliability, and ruggedness of the procedure, 2 trials were conducted with prepared PROFIT kits, containing all necessary materials, on a wide variety of meat products as blind screen (unknown) samples. One trial was conducted in our laboratories under controlled testing conditions resembling those of a field situation. The other trial was an actual field trial performed by visiting 3 individual meat processing plants in Baltimore, MD, and analyzing sample products collected by federal meat inspectors at those establishments. The results of these trials are shown in Table 1. Of the 66 samples examined during these trials, 49 contained poultry proteins and all of these gave positive PROFIT reactions, thereby demonstrating 100% accuracy of the procedure. All remaining samples (17) devoid of poultry proteins failed to give positive reactions as expected. The results of these trials established the reliable and practical field application of PROFIT for a wide variety of commercial meat products with a minimum expenditure of time and effort in the testing procedure.

Discussion

We have successfully developed a practical serological screen testing system for field use to determine the presence of poultry proteins in diverse sample populations. The highly economical nature of the test procedure, even after it becomes available commercially as anticipated in the near future, should allow any type of inspection program to adequately use the procedure to screen large sample numbers to assure compliance with regulations concerning the detection or verification of poultry species composition in meat products or whole meat (poultry) pieces. We know of no other reported poultry screening test system which is as easy to perform and interpret or has equivalent long-term stability characteristics.

Important to the successful development of the described procedure was the production of a suitable antiserum. We elected to base our antiserum on an anti-albumin species system, as previously reported by Kamiyama et al. (10) for specific application to meat species other than poultry. This was done to take advantage of the commercial availability of highly purified albumins which are good immunogens and are present in a native state to a significant degree in finished raw meat products and can therefore serve as convenient markers on which to base a sound species serological detection system. The use of albumin also facilitates preparation of an easily standardizable, stable, test reference antigen. The albumin anti-albumin basis of our system, however, is the likely reason that products containing significant chicken egg white content also give a positive reaction (Table 1). This fact should be taken into consideration when processed meat products that may contain chicken eggs as an ingredient are analyzed.

We also chose to prepare antiserum having a broad poultry specificity range for our intended needs and purposes. We employed a large animal host for greater antiserum yield. Other investigators who wish to establish their own testing system may not necessarily choose to do the same. Smaller animals such as rabbits could also be used for suitable antiserum production if yield is not an important consideration, because there does not appear to be any natural cross-relatedness between poultry and red meat proteins. The intended broad-based poultry specificity range of our system would obviously require the use of other available laboratory procedures (2–8) if the exact poultry species were required to be identified for a given sample. It also appears possible that a very specific system could be developed, by those individuals requiring it, by preparing with some effort a mono-specific poultry species antiserum. We do not however anticipate that any problems will arise with the intended use of our system on the more commonly occurring chicken and turkey species products due to its broad poultry specificity range. The potential exists for the specific application of PROFIT in special cases where needed for the detection of the less commonly occurring poultry species.

During the course of this investigation we briefly examined the suitability of commercially available rabbit anti-chicken and anti-turkey serum prepared from immunizations with poultry whole serum. We found these to be capable of functioning properly but at a higher cost for reagent disc preparation.

It is expected that the use of PROFIT as a first-line inspection safeguard will discourage and diminish the significant economic and potential health problem of adulteration of meat products with poultry. Its use will also allow exporting nations to meet, in part, equivalent meat and poultry inspection systems and/or laboratory services of an importing nation in an economically feasible manner. In specific cases where legal action is contemplated with regard to an adulteration or substitution, PROFIT-positive results may still be confirmed with the traditional Ouchterlony immunodiffusion technique (3) or by isoelectric focusing (7) if desired.

Acknowledgments

The authors thank Charles P. Lattuada and Cheryl L. Schwartz of Granite Diagnostics Inc., for the production and supply of goat anti-poultry serum used for the development of the reported procedure.

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PESTICIDE FORMULATIONS

Gas Chromatographic Determination of Fensulfothion in Formulations: Collaborative Study

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A collaborative study was conducted on a gas chromatographic method for determination of fensulfothion. Eleven laboratories analyzed 2 technical and two 6 lb/U.S. gal. spray concentrate samples. In the analysis, samples are dissolved in methylene chloride which contains 4-chlorophenyl sulfoxide as an internal standard, and solutions are injected into a gas chromatograph equipped with an OV-330 column. Within-laboratory repeatability was 0.79% for technical product and 0.37% for the spray concentrate samples, with coefficients of variation of 0.88 and 0.58%, respectively. Among-laboratories reproducibility was 0.81% for technical product and 0.53% for the spray concentrate, with coefficients of variation of 0.91 and 0.84%, respectively. The method has been adopted official first action.

Fensulfothion, *O,O*-diethyl *O*-[4-(methylsulfinyl)phenyl] phosphorothioate, has been used as an insecticide and nematocide. It is the active ingredient in Danasit® (registered trademark of the parent company of Farbenfabriken Bayer, GmbH, Leverkusen, FRG).

AOAC has adopted a liquid chromatographic method for determination of fensulfothion in formulations (1, 2). We decided to develop and test a gas chromatographic (GC) method for fensulfothion. Several GC methods have been published (3, 4) for determining residues of fensulfothion by using OV-17, OV-210, OV-101, DC-200, and DEGS columns. Those methods attempt to separate fensulfothion from plant material and other pesticides rather than from its own impurities. We have found that OV-330 is an efficient column packing for separating fensulfothion and its impurities. This paper describes the results of a collaborative study of a GC method, using an OV-330 column.

Collaborative Study

Standard fensulfothion (88.7%), a practice sample, 4-chlorophenyl sulfoxide for use as an internal standard, 2 different samples of technical fensulfothion and 2 different samples of 6 lb/U.S. gal. spray concentrates were sent to each collaborator. Both sample pairs were close but not identical in fensulfothion content, as recommended by Youden and Steiner (5). Each collaborator was also supplied with 5% OV-330 column packing and was asked to pack a column. The collaborators were requested to make a single GC determination for each sample from duplicate injections, and to report area integration and peak height measurements. The collaborators were requested to submit the raw data and chromatograms to the Associate Referee.

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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.* (1986) **69**, March issue.

Fensulfothion in Pesticide Formulations

Gas Chromatographic Method

First Action

(Method is suitable for tech. and liq. formulations of fensulfothion.)

6.B28 ***Principle***

Sample is dissolved in CH_2Cl_2 contg 4-chlorophenyl sulfoxide as internal std, and fensulfothion is detd by gas chromatgy.

6.B29 ***Apparatus***

(a) *Gas chromatograph.*—Equipped with flame ionization detector (FID). Temps—column 225°, injection port 250°, detector 250°; carrier gas 30–40 mL/min (either He or N); air and H flows as recommended for FID; sample size 2.0 μL; retention times (min)—internal std 4.0, fensulfothion 5.5. Adjust parameters to cause fensulfothion to elute in 5–6 min, but do not use column temp. >240°. If internal std and fensulfothion peaks are not completely sepd, repack column.

(b) *Column.*—0.9 m (3 ft) or 1 m × 2 mm (id) glass column packed with 5% OV-330 on 80–100 mesh Chromosorb WHP (Supelco). Condition newly packed columns 8–16 h at 240° before use.

6.B30 ***Reagents***

(a) *4-Chlorophenyl sulfoxide.*—Aldrich Chemical Co., Cat. No. 12,104-5, or equiv. that contains no impurities eluting at retention time of fensulfothion.

(b) *Internal std soln.*—Weigh 1.0 g 4-chlorophenyl sulfoxide, dissolve in 1 L CH_2Cl_2, and mix well. Keep tightly stoppered.

(c) *Fensulfothion reference std soln.*—Accurately weigh amt of ref. std (Mobay Chemical Corp.) contg ca 100 mg fensulfothion into ca 100 mL glass bottle. Add by pipet 50.0 mL internal std soln. Stopper and mix well.

6.B31 ***Preparation of Sample***

Accurately weigh sample contg ca 100 mg fensulfothion into glass bottle (ca 100 mL). Pipet in 50.0 mL internal std soln. Stopper and mix well.

6.B32 ***Determination***

Make repetitive 2 μL injections of fensulfothion ref. std soln until response is stable and ratios of fensulfothion peak area to internal std peak area for successive injections agree within 1% of their mean. Peak ht may be substituted for peak area.

Make duplicate 2 μL injections of each sample. Response ratios (R) for fensulfothion internal std for 2 sample injections must agree ± 1% of their mean. If not, repeat detn, starting with std injections. After every 4–6 sample injections and after last sample injection, make 2 injections of fensulfothion std soln. Av. std soln ratios preceding and following sample must be ± 1.0% of mean; otherwise, repeat series of injections.

6.B33 ***Calculation***

Calc. ratios for each injection. Average 2 sample ratios and 4 std ratios (std injections immediately before and after sample injections).

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

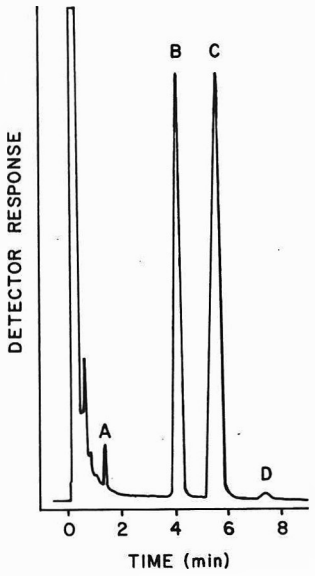

Figure 1. Typical chromatogram from collaborative study showing fensulfothion sulfide (A), 4-chlorophenyl-sulfoxide (B), fensulfothion (C), and fensulfothion sulfone (D).

where R and R' = av. sample and std ratios (fensulfothion peak/internal std peak), resp.; W and W' = mg sample and std, resp.; and P = % purity of fensulfothion std.

Results and Discussion

A linearity test was conducted by weighing 100, 200, and 300 mg fensulfothion into separate bottles, pipetting 100 mL of internal standard into each, and comparing the peak area ratios of the 100 and 300 mg standards to the 200 mg standard. The ratio for the 100 mg standard was 100.9% of theoretical and for the 300 mg standard 99.8% of theoretical, indicating a wide range of linear response.

A recovery test was carried out by accurately weighing standard fensulfothion into a spray concentrate blank (consisting of all of the spray concentrate ingredients except fensulfothion), and then determining fensulfothion by the GC method. Recoveries on 3 mixtures were 100.4, 99.9, and 99.5% of theoretical, indicating that the spray concentrate ingredients do not interfere with the GC determination of fensulfothion.

Figure 1 shows an actual chromatogram from the study. There are 2 major impurities: peak A is fensulfothion sulfide (CH_3S- group in para position on the ring) and peak D is fensulfothion sulfone (CH_3SO_2- group in the para position).

Eleven collaborators analyzed 4 samples. Collaborator 1 conditioned the column at 250°C for 3 days, which was excessive and caused a large amount of column bleed. Short retention times and tailing peaks resulted, with peak D in the tail of the fensulfothion peak. Collaborator 11 evidently had instrument problems with several chromatograms, which showed large baseline drops after the fensulfothion peak, and the duplicate injections of the standards did not agree to within 1% of their mean as required by the method. The data from Collaborators 1 and 11 were eliminated from further consideration. One sample pair from Collaborator 10 was identified as an outlier by the Dixon test and therefore was rejected. The remaining data showed good agreement between collaborators. (Table 1). Repeatability coefficients of variation were 0.88% for technical product and 0.58% for spray concentrate samples. Reproducibility coefficients of variation were 0.91% and 0.84% for technical and spray concentrate samples, respectively.

Collaborators 1 and 11, whose results were rejected, commented about asymmetric peaks or poor reproducibility. The remaining collaborators either had favorable comments or made no comment.

Of the 9 collaborators with acceptable chromatograms, 8 reported peak area data, 4 reported both peak area and peak height data, and 1 reported only peak height data. With only 4 collaborators reporting parallel peak area and peak height data, data were insufficient for a complete statistical evaluation of peak height vs peak area; however, the means for the 4 samples by the 4 collaborators using peak area were 89.33, 89.38, 63.42, and 62.74%. By peak height, means were 89.59, 88.87, 63.56, and 62.90%. The difference, which is within the reproducibility of the method, indicates no significant difference between the 2 methods of quantitation.

All collaborators used glass columns of 2 mm id except Collaborator 7 who used a 2 ft × 4 mm id glass column, with good results. Collaborator 2 used a 6 ft column but even with a very high flow rate had retention times of 7.5 and 10.5 min, unnecessarily long for routine analysis. The remaining collaborators used 3 or 4 ft columns. Column temperature ranged from 225 to 250°C. A column length of 3 ft or 1 m is recommended to keep the column temperature at or below 240°C to minimize column bleed.

Recommendation

It is recommended that the gas chromatographic method for determination of fensulfothion in technical and liquid formulations be adopted official first action. The preferred column length is 3 ft (0.9 m) or 1 m.

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Edwin W. Balcer, State Dept of Agriculture, St. Paul, MN

Oliver O. Bennett, Jr, State Dept of Agriculture Laboratory, Topeka, KS

Table 1. Collaborative results on fensulfothion (%) in technical product and formulation as closely matched pair samples

	Technical		6 lb/U.S. gal. SC	
Coll.	A	B	A	B
1[a]	—	—	—	—
2	88.29	88.29	63.20	62.47
3	89.93	89.38	63.41	62.60
4	90.17	88.46	64.01	62.76
5	88.93	88.76	62.79	62.02
6	88.00	88.82	63.07	62.40
7	89.53	87.88	63.45	62.20
8	88.12	89.61	63.46	63.84
9	89.05	90.08	62.79	61.76
10[b]	89.96	90.02	65.94[c]	65.24[c]
11[d]	—	—	—	—
Mean	89.11	89.03	63.27	62.51
S_o	0.787		0.365	
S_L	0.187		0.380	
S_x	0.809		0.527	
CV_o,%	0.88		0.58	
CV_x,%	0.91		0.84	

[a]Excessive column bleed—data not included.
[b]Peak height measurements.
[c]Rejected by Dixon test.
[d]Instrument problems—data not included.

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Liquid Chromatographic Method for Determination of Oxythioquinox in Technical and Formulated Products: Collaborative Study

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A liquid chromatographic method for determination of oxythioquinox (Morestan®) in oxythioquinox technical and formulated products has been developed and collaboratively studied in 14 laboratories. Samples are dissolved in chloroform containing *n*-valerophenone as an internal standard, diluted with acetonitrile, and analyzed by reverse phase chromatography. Collaborators analyzed blind duplicate samples of oxythioquinox technical and 25 WP. Coefficients of variation were 1.06 and 1.72% for the technical and 25 WP samples, respectively. The method has been adopted official first action.

Oxythioquinox (Morestan®), 6-methyl-1,3-dithiolo[4,5-*b*]quinoxalin-2-one, is available as a 25% wettable powder (25 WP) formulation. Oxythioquinox is an insecticide-acaricide-fungicide, and is very effective in the control of aphids, whiteflies, powdery mildew, and resistant and nonresistant strains of several mite species on most deciduous fruits and ornamentals.

Several methods for the determination of oxythioquinox residues have been reported using direct gas chromatography (1–6) and also liquid chromatography with fluorescence detection (7–9). Two colorimetric methods have been reported for the analysis of oxythioquinox samples (6, 10). The present report describes a collaborative study of a liquid chromatographic (LC) method with ultraviolet detection. 1-Phenyl-1-pentanone (*n*-valerophenone) is used as an internal standard.

Collaborative Study

The LC method was sent to 14 collaborators. Each collaborator received blind duplicate subsamples of the technical material and the formulation, a reference standard, and the internal standard. The study was designed according to suggestions given by Youden and Steiner (11).

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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.* (1986) **69**, March issue.

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

Oxythioquinox in Pesticide Formulations

Liquid Chromatographic Method

First Action

AOAC-CIPAC Method

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

6.B34 ***Principle***

Sample with 1-phenyl-1-pentanone internal std is extd with CH_3CN, and oxythioquinox is detd by reverse phase liq. chromatgy.

6.B35 ***Apparatus and Reagents***

(a) *Liquid chromatograph.*—Able to generate >10 MPa (>1430 psi) and measure *A* at 280 nm. Operating conditions: column temp. ambient; flow rate 2 mL/min (ca 5 MPa); chart speed 0.5 cm/min; injection vol. 10 μL; *A* range 0.320 AUFS; retention times: 1-phenyl-1-pentanone ca 3.1 min, oxythioquinox ca 5.4 min. Pump LC mobile phase thru column until system is equilibrated (flat baseline). Allow each injection ca 7 min run time, then pump CH_3CN ca 4 min to remove impurities. Pump LC mobile phase ca 4 min, allowing system to re-equilibrate before next injection.

(b) *Chromatographic column.*—250 × 4.6 mm id packed with ≤10 μm C18 bonded silica gel.

(c) *Acetonitrile.*—LC grade or distd in glass (Burdick & Jackson Laboratories, Inc., or equiv.).

(d) *Chloroform.*—Spectrophtric grade or equiv.

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

(f) *1-Phenyl-1-pentanone (n-valerophenone) internal std soln.*—1 g/100 mL $CHCl_3$.

(g) *Reference std oxythioquinox.*—Mobay Chemical Corp.

(h) *Water.*—LC grade or distd in glass (Burdick & Jackson Laboratories, Inc., or equiv.).

(i) *LC mobile phase*—CH_3CN–H_2O (80 + 20).

Table 1. Collaborative results of the LC analysis of oxythioquinox technical and formulation as blind duplicate samples

	Technical		25 WP	
Coll.	A	B	A	B
1	96.14	95.30	25.99	26.04
2	95.71	94.99	25.53	25.63
3	92.43	92.32	26.57	26.88
4	94.82	96.07	25.88	26.24
5	94.66	95.05	25.48	25.56
6	94.40	95.08	25.57	25.77
7	96.03	95.56	27.17	26.76
8	94.90	95.04	25.88	26.04
9	94.16	94.88	25.47	25.57
10	94.38	95.07	26.34	26.16
11	93.38	93.07	25.87	25.89
12	94.90	94.96	26.05	26.17
13	96.28	95.26	25.75	25.89
14	94.46	94.60	25.62	25.69
Mean	94.78		25.98	
S_x	1.001		0.447	
S_o	0.455		0.225	
S_L	0.947		0.418	
CV_x, %	1.056		1.720	
N	28		28	

6.B36 ***Preparation of Standard***

Accurately weigh ca 100 mg ref. std into 100 mL vol. flask. Pipet 10 mL internal std soln into flask and swirl to mix. Add ca 50 mL CH_3CN, sonicate 4 min, dil. to vol. with CH_3CN, and mix well. Filter portion of soln for LC analysis.

6.B37 ***Preparation of Sample***

Accurately weigh amt of sample contg ca 100 mg oxythioquinox into 100 mL vol. flask. Pipet 10 mL internal std soln into flask, and swirl to mix. Add ca 50 mL CH_3CN, sonicate 4 min, dil. to vol. with CH_3CN, and mix well. Filter portion of soln for LC analysis.

6.B38 ***Determination***

Adjust operating parameters to elute oxythioquinox in 5.0–5.9 min. Adjust injection size and attenuation to give largest possible on-scale peaks. Make repetitive injections of ref. std soln and calc. response ratios (R) = oxythioquinox peak area (or ht)/internal std peak area (or ht). Response ratios must agree ±1%. Average duplicate response ratios obtained with ref. std soln.

Inject duplicate aliquots of each sample soln. Average response ratios for each sample soln. Response ratios must agree ±1%. If not, repeat detn, starting with std injections.

Re-inject ref. std soln twice. Average response ratios of stds immediately preceding and following sample injections. These must agree ±1%. If not, repeat detn.

6.B39 ***Calculation***

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

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

Results and Discussion

A complete set of results was received from each of the 14 collaborators (Table 1). The collaborators used a variety of equipment to perform the analyses: 4 brands of liquid chromatographs, 4 brands of injectors, and 8 types of columns including Du Pont Zorbax ODS, Whatman Partisil ODS and Partisil ODS-3, Varian Instrument Micropak MCH 5 and 10, Alltech Associates Alltech-C18, E. Merck Science Lichrosorb RP-18, and Waters Associates Radial-PAK μBondapak C-18. Sample volumes injected varied from 5 to 21 μL. The pressures obtained were 2.3 to 27 MPa, and flow rates from 1 to 2 mL/min were used. Nine collaborators determined response ratios by using data systems; 5 collaborators used peak height measurements.

Previous experience with one of the colorimetric methods has shown that it gives values very near those of this LC method (6). However, the colorimetric method is subject to interference from a major oxythioquinox impurity. This impurity, 2,10-dimethyl[1,4]dithiino[2,3-*b*:5,6-*b'*]diquinoxaline (and/or its 2,9 isomer) can give an erroneously low bias to that colorimetric method by affecting accurate measurement of the oxythioquinox minima.

In addition to this impurity, shown as dithioether in Figure 1, this LC method resolves all known impurities in oxythioquinox. The solvent flush specified is necessary to elute the dithioether and sulfur quickly to avoid interferences in subsequent injections.

The internal standard is prepared in chloroform to ease the extraction and dissolution of oxythioquinox. Although oxythioquinox is soluble at these concentrations in acetonitrile, the use of chloroform eliminates an extended shake step and has no adverse effects on the resolution or reproducibility of the method.

An attempt was made originally to develop the separation by using a methanol–water mobile phase. This proved unac-

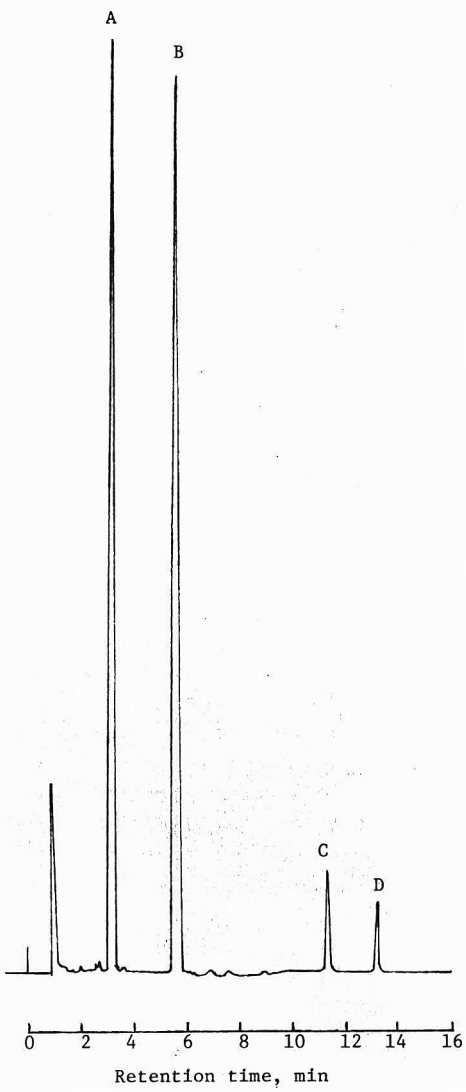

Figure 1. LC chromatogram of valerophenone internal standard (A), oxythioquinox (B), dithioether compound (C), and sulfur (D).

ceptable due to the apparent decomposition of oxythioquinox in methanol to 6-methyl-2,3-quinoxalinedithiol.

As 2 collaborators pointed out, volumetric flasks are not mandatory with the use of internal standards. The method stipulates these only from the practical standpoint that such glassware is commonly available in analytical laboratories.

Recommendation

It is recommended that the LC method for determination of oxythioquinox technical and in formulation be adopted official first action as an AOAC-CIPAC method.

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VETERINARY ANALYTICAL TOXICOLOGY

Determination of Diagnostic Levels of Arsenic in Animal Tissue: Collaborative Study

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Collaborators: G. H. D'Andrea; R. J. Audette; B. Colvin; G. George; M. Gerlach; A. V. Jain; J. Reagor; J. E. Roof; H. M. Stahr; B. Tipton; P. Weaver; J. Willard

The method chosen for this collaborative study is a modification of the AOAC method for As residues, 41.009–41.012. The tissue is dry-ashed overnight at 500°C, and then dissolved in dilute HCl. The solution is diluted and an aliquot is reacted with zinc metal to evolve arsine gas. The gas is trapped in AgDDC solution and As is quantitated at 540 nm. Nine collaborating laboratories performed single analyses on 4 blind duplicate pairs of bovine liver samples which were spiked at 0, 4.3, 10.8, or 21.6 mg As/kg liver. A National Bureau of Standards control (SRM 1566 Oyster Tissue, 13.4 ± 1.9 mg As/kg) and a 1000 mg As/L standard were also submitted to the collaborators. Intralaboratory coefficients of variation ranged from 7.7 to 17.8%; interlaboratory coefficients of variation ranged from 10.9 to 19.0%. The method has been adopted official first action.

Arsenic has been and will continue to be a major source of animal poisonings because of its diversified use as an insecticide, herbicide, and defoliant (1). A survey of laboratories for methods being used to determine high levels of As in animal tissues indicated the need for an official method (2).

The method chosen for study is a modification of the AOAC method (3) for As residues in animal tissue **(41.009–41.012).** Modifications were made to accommodate the high As levels found in tissues of poisoned animals.

Collaborative Study

The method was submitted to 12 laboratories with an external control sample, 4 blind duplicate pairs of spiked or blank bovine liver samples, and a 1000 mg As/L standard. Each laboratory was asked to keep the samples frozen until analysis and to blend the samples thoroughly before weighing. They were also asked to analyze each sample only once.

Preparation of Collaborative Samples

The samples for the collaborative study were prepared as follows: Fresh bovine liver tissue (containing background level of 0.05 mg As/kg) was cut into cubes (cu. cm), the cubes were put individually into liquid nitrogen (LN_2), and then ground with additional LN_2 in a Stein mill. After the liver was thawed, 200 g portions were transferred to a Waring blender. An aqueous solution of 863 μg/mL of As in the form of arsanilic acid was used to spike the liver at various levels. After spiking, the liver was blended 5 min at moderate speed and then analyzed by atomic absorption spectrophotometry (AAS) (4) to ensure the accuracy of the spiking procedure. The theoretical and actual values of the 4 pairs of blind duplicates are shown in Table 1.

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The recommendation of the Associate Referee was approved by the General Referee and the Committee on Feeds, Fertilizers, and Related Materials and was adopted by the Association. See the General Referee and Committee reports, *J. Assoc. Off. Anal. Chem.* (1986) **69**, March issue.

Arsenic in Liver Tissue
Spectrophotometric Method
First Action

49.B01 ***Principle***

Liver tissue is dry-ashed overnight at 500°, ash is dissolved, and portion is reacted with Zn metal to evolve arsine gas. Arsine is trapped and As is detd spectrophtric.

49.B02 ***Apparatus and Reagents***

(a) *Hydrochloric acid.*—3N.

(b) *Copper sulfate.*—Anhyd., powd (J.T. Baker Chemical Co., or equiv.).

(c) *Magnesium oxide–magnesium nitrate slurry.*—Suspend 7.5 g MgO and 10.5 g $Mg(NO_3)_2.6H_2O$ in enough H_2O to make 100 mL. Agitate vigorously before adding to sample.

(d) *Stannous chloride soln, 20% (w/v).*—Dissolve 20 g As-free $SnCl_2.2H_2O$ in HCl and dil. to 100 mL with HCl.

(e) *Silver diethyldithiocarbamate (AgDDC) soln.*—Dissolve 0.50 g AgDDC salt in pyridine and dil. to 100 mL with pyridine. Mix and store in amber bottle. Reagent is stable several months at room temp. (Fisher Scientific Co., Cat. No. S-666, or equiv.).

(f) *Arsenic std solns.*—(*1*) *Stock soln.*—500 μg/mL. Accurately weigh 0.660 mg NBS Ref. Std As_2O_3, or equiv., dissolve in 25 mL 2N NaOH, and dil. to 1 L with H_2O. (*2*) *Intermediate soln.*—10 μg/mL. Transfer 2 mL stock soln to 100 mL vol. flask, and dil. to vol. with H_2O. (*3*) *Working soln.*—2 μg/mL. Transfer 10 mL intermediate soln to 50 mL vol. flask and dil. to vol. with H_2O.

(g) *External control.*—Std Ref. Material (SRM) 1566 Oyster Tissue (13.4 ± 1.9 mg As/kg) or equiv.

(h) *Potassium iodide soln, 15% (w/v).*—Dissolve 15 g KI in H_2O and dil. to 100 mL.

(i) *Zinc.*—Shot contg <0.00001% As (Fisher Scientific Co., No. Z-12).

(j) *Distillation apparatus.*—See **41.009(e)**. Use 125 mL erlenmeyer instead of 250 mL. Use narrow test tube as receiver and submerge delivery tube in AgDDC soln.

49.B03 ***Preparation of Standard Curve***

Transfer 0.5, 1.0, 3.0, 6.0, and 10.0 mL aliquots of working soln corresponding to 1, 2, 6, 12, and 20 μg As to sep. 125 mL erlenmeyers. Dil. to 50 mL with 3N HCl. Carry these solns thru distn procedure. Plot *A* at 540 nm on ordinate vs μg As on abscissa. Det. best fitting straight line, using all 5 points, by method of least squares.

49.B04 ***Preparation of Sample***

Blend tissue in high-speed blender until completely homogeneous. Accurately weigh 2.00 g tissue into 30 mL Coors crucible. Analyze

Table 1. Arsenic concentration (mg/kg) of prepared liver samples

Sample pair	Theoretical	AAS[a]
A, H	21.6	21.1
B, G	10.8	9.9
C, F	4.3	4.4
D, E	nil	0.046

[a]As determined by hydride AAS.

Table 2. Collaborative results on As (mg As/kg liver) in blind duplicate sample pairs

	Sample							
Lab.	D	E	C	F	B	G	A	H
1	nil	nil	3.5	6.3	9.5	12.1	23.8	18.7
2	nil	nil	4.1	4.0	13.5	10.4	18.5	NR[a]
3	nil	nil	4.7	5.1	12.9	12.4	23.6	22.0
5	nil	nil	4.5	4.8	11.0	10.8	20.8	20.6
7	nil	nil	3.5	3.4	9.6	9.9	21.8	22.0
8	nil	nil	3.8	3.2	7.6	13.9	NR[a]	15.7
9	nil	nil	4.7	5.6	11.0	12.0	—[b]	—[b]
11	nil	nil	3.7	4.7	8.6	8.6	19.2	19.2
12	nil	nil	4.4	3.8	12.2	13.0	21.2	22.4

[a]Results not received.
[b]Results on samples A and H excluded on basis of analysis (see text).

Table 3. Statistical data for collaborative study of As in liver samples[a]

	Sample pair			
Statistic	C, F	B, G	A, H	D, E
Mean, mg/kg[b]	4.3	11.1	20.7	nil
Repeatability				
SD_0[c]	0.77	1.80	1.58	—
CV_0,%	17.8	10.3	7.7	—
Reproducibility				
SD_x[d]	0.82	1.83	2.26	—
CV_x	19.0	16.5	10.9	—
% Recovery	100	103	96	—

[a]Lower limit of detection is 1 mg As/kg liver.
[b]Nine collaborative results.
[c]Includes within-lab. and day-to-day variation.
[d]Interlaboratory variation.

one external control with each set of 10 samples or fraction thereof. Add 5 mL well mixed $MgO/Mg(NO_3)_2.H_2O$ slurry and mix thoroly with stirring rod. Prep. blank by adding 5 mL well mixed slurry to sep. crucible and carrying it thru subsequent steps in procedure. Dry samples, controls, and blank to apparent dryness on hot plate or in drying oven at <100°. Cover each crucible with watch glass and place in cold muffle furnace. Set furnace temp. at 250° for 3 h; then gradually increase temp. to 500° and leave overnight.

Cool crucibles to room temp., moisten residue with 5 mL H_2O, and transfer quant. to 50 mL vol. flask with 3N HCl. Dil. to vol. with 3N HCl and mix well. Transfer 25 mL aliquot to 125 mL erlenmeyer and dil. to 50 mL with 3N HCl.

49.B05 ***Distillation***

Add 2 mL 15% KI soln and swirl. Add 1 mL $SnCl_2$ soln and swirl. Cool flasks in freezer or ice bath 45 min or until samples reach 4°. Pipet 6 mL AgDDC soln into narrow receiver test tube, one for each std, external control, sample, and blank. Have all parts of distn app. ready for immediate assembly. Quickly add 10 g Zn shot and pinch of Cu_2SO_4 to erlenmeyer, assemble app., and distil 1 h at room temp. Det. *A* at 540 nm for blank, external control, sample, and std AgDDC solns in suitable spectrphtr. Subtract blank reading from sample and control, and det. mg As/kg directly from std curve. External control results must fall within accepted range (95% confidence limit) for all results to be valid.

Results and Discussion

Complete results were received from 10 of the participating laboratories. However, 1 set of results was not returned in time for statistical analysis, so only 9 are included in the summary of results shown in Table 2.

Most collaborators commented favorably. Several mentioned the importance of cooling the reaction vessel to 4°C. A previous unsuccessful collaborative study (5) did not specify this step and many collaborators felt that losses occurred due to the uncontrollable rate of arsine gas evolution.

One collaborator suggested decreasing the number of standards and using a calculator instead of a standard curve. This modification would allow more samples to be run with each set and calculations would be expedited.

Most laboratories submitted standard curves with their results. To facilitate calculations, a 1 g equivalent aliquot of sample is used for the determination. This permits reading mg As/kg sample directly from the standard curve.

Results from Laboratory 9 for samples A and H were excluded because they calculated results for those 2 samples at a point in excess of the highest standard on the standard curve. All other results were used for calculation of the statistical summary (6) shown in Table 3. Mean recoveries were 100, 103, and 96% for the 3 pairs spiked 4.3, 10.8, and 21.6 mg/kg, respectively. For those samples with readings below 1 mg/kg, the collaborators were requested to report "nil." All collaborators correctly analyzed the blank samples and there were no false positive results. For diagnostic purposes, concentrations of 1 mg/kg or less are of little value (7). The interlaboratory variances are consistent with previous work (8, 9).

The use of an external control was invaluable in giving confidence and credibility to the study. SRM 1566 from the National Bureau of Standards (NBS) is actually oyster tissue, which is not a preferred matrix match, but is the only biological SRM offered which has a level of arsenic in the diagnostic range. Not only were the collaborators requested to run the SRM as a control with each set of samples, but also as a practice sample to familiarize themselves with the method before starting the actual samples. The average value from 10 laboratories for arsenic in the SRM was 12.7 ppm ± 1.8, which is within the confidence limits of 13.4 ppm ± 1.9 set by NBS.

Recommendation

The Associate Referee recommends that this method for determination of diagnostic levels of As in animal tissue be adopted official first action.

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EXTRANEOUS MATERIALS

Colorimetric Determination of Alkaline Phosphatase as Indicator of Mammalian Feces in Corn Meal: Collaborative Study

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In the official method for rodent filth in corn meal, filth and corn meal are separated in organic solvents, and particles are identified by the presence of hair and a mucous coating. The solvents are toxic, poor separation yields low recoveries, and fecal characteristics are rarely present on all fragments, especially on small particles. The official AOAC alkaline phosphatase test for mammalian feces, 44.181–44.184, has therefore been adapted to determine the presence of mammalian feces in corn meal. The enzyme cleaves phosphate radicals from a test indicator/substrate, phenolphthalein diphosphate. As free phenolphthalein accumulates, a pink-to-red color develops in the gelled test agar medium. In a collaborative study conducted to compare the proposed method with the official method for corn meal, 44.049, the proposed method yielded 45.5% higher recoveries than the official method. Repeatability and reproducibility for the official method were roughly 1.8 times more variable than for the proposed method. The method has been adopted official first action.

The official AOAC method for detecting rodent filth in corn meal (1) separates fecal matter and corn meal by differences in their specific gravities in chloroform and carbon tetrachloride. In these organic solvents, the fecal particles should sink while the corn meal floats. However, because the fecal particles tend to float throughout the solvents instead of settling to the bottom, separation and recovery are usually not achieved. Any suspect particles recovered from the corn meal can be identified as fecal only if hair and a mucous coating are present. Because these characteristics are seldom present in very small particles, positive identification can rarely be made.

An alternative method, which is a modification of the official AOAC enzymatic test for mammalian feces (2), has been developed. Feces and corn meal are separated by differences in their specific gravities in a hot liquid test agar. The corn meal sinks into the agar and the fecal particles remain on the surface. Alkaline phosphatase, a constituent of mammalian excreta, identifies fecal particles by cleaving phosphate radicals from the substrate/pH indicator phenolphthalein diphosphate in the test agar medium, which is colorless initially but changes to pink and sometimes red as both phosphate radicals are liberated from phenolphthalein diphosphate. The gelled agar in the test medium retards diffusion of alkaline phosphatase, keeping the concentration high, and a pink-to-red spot appears in the agar, surrounding each fecal particle. The number of positive spots observed is tabulated and reported as number of fecal particles per 10 g of test portion. This enzymatic analysis can detect particles as small as 250 μm. No toxic reagents are used, and the method is relatively simple and rapid to perform (1.5 h total analytical time).

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Collaborative Study

Corn meal free of fecal contamination was weighed into 50 g portions for analysis by the official method and 10 g portions for the proposed method. Rodent fecal particles were mixed in to provide spike levels of 1, 20, 30, and 50. Except for those with only 1 particle, the test portions contained 1 additional particle larger than the rest to serve as a positive control. Control particles used for the official method were known to contain at least 1 hair.

Each of 12 collaborators was sent 2 sets of corn meal samples, 1 set for analysis by the official method and the other by the proposed method. Each set consisted of duplicates of the 4 spike levels. Two practice test portions spiked with 20 and 30 particles were provided for each method. The collaborators also received indicator phenolphthalein diphosphate, copies of the methods, instructions and cautions, and forms for recording results, questions, and comments. Expected minimum recoveries for the practice test portions were given, and collaborators were requested to call if these minimum results were not achieved.

Mammalian Feces in Corn Meal
Alkaline Phosphatase Detection Method
First Action

44.B01 ***Principle***

Intestinal tract of most mammals contains alk. phosphatase enzyme. Enzyme at test pH and temp. splits phosphate radicals from substrate/pH indicator phthln diphosphate to produce light pink to red-purple color from free phthln.

44.B02 ***Apparatus***

(a) *Hot water bath.*—Maintained at 42° ± 1°.

(b) *Hot plate stirrer and 41 mm ovoid stirring bar.*—Fisher 1451158A or equiv.

(c) *Petri dishes.*—Plastic disposable, 150 × 20 mm or 150 × 15 mm (Falcon 1058 or plastic/glass equiv.).

(d) *Weighing boats.*—8.1 × 8.1 × 1.9 cm, 100 mL capacity (Fisher Scientific Co., Cat. No. 02210B, or approx. size equiv.).

44.B03 ***Reagents***

(a) *Magnesium chloride soln.*—Dissolve 0.203 g $MgCl_2.6H_2O$ and dil. to 500 mL with H_2O. Indefinite shelf life.

(b) *Stock test reagent.*—Dissolve 19.0 g borax ($NaB_4O_7.10H_2O$) and 6.28 g anhyd. Na_2CO_3 in 1 L H_2O with stirring. Add 0.94 g phthln diphosphate and stir while adding 2 mL $MgCl_2$ soln. Prepn is stable ca 4 months at room temp. Soln should be colorless and ca pH 9.5. Discard if not colorless. Degraded phthln diphosphate produces pink color in reagent. Store phthln diphosphate in desiccator below 0°. (Phthln diphosphate, Sigma P 9875.)

(c) *Liquid test agar.*—Prep. fresh before using, 150 mL per 10 g sample to be analyzed. Measure equal vol. of stock test reagent, (b) (half of total test agar vol. needed), and H_2O into sep. appropriate size beakers. Beaker for H_2O must be large enough to accommodate

Table 1. Collaborative results for recovery of fecal particles

	Official method[a] Spiked level								Proposed method[b] Spiked level							
	1		20		30		50		1		20		30		50	
Coll.	Sub 1	Sub 2	Sub 1	Sub 2	Sub 1	Sub 2	Sub 1	Sub 2	Sub 1	Sub 2	Sub 1	Sub 2	Sub 1	Sub 2	Sub 1	Sub 2
A	20[c]	9[c]	25[c]	16[c]	6[c]	13[c]	5[c]	9[c]	1	5	14	8	11	19	15	30
B	0	1	1	2	1	1	1	1	0	2	12	7	24	11	27	30
C	1	1	2	2	2	1	1	2	2	1	15	14	18	14	34	26
D	0	1	0	3	1	0	1	2	7[c]	2[c]	5[c]	5[c]	10[c]	11[c]	16[c]	8[c]
E	0	0	0	0	0	0	0	0	1	3	7	11	11	17	11	18
F	1	2	1	1	2	2	0	2	0	2	5	9	15	13	37	26
G	5[d]	2[d]	2	3	2	1	4	2	1	0	14	13	25	11	24	35
H	1	1	2	1	2	1	2	2	8[d]	8[d]	13	21	33[d]	32[d]	38	39
I	1	2	4	2	2	2	1	4	1[d]	10[d]	10	8	11	12	20	27
J	1	1	1	1	1	1	1	1	1	1	8	4	12	18	30	16
K	1	0	1	1	1	1	1	1	1	2	6	4	8	9	20	22
Statistical analysis:																
$\bar{X}$	0.8		1.5		1.2		1.4		1.4		10.1		14.4		26.2	
$\bar{X}$, %	80.0		7.5		4.0		2.9		140.0		50.5		48.0		52.4	
SD																
Repeatability	0.5		0.9		0.4		1.0		1.4		3.0		5.4		6.5	
Reproducibility	0.6		1.0		0.7		1.1		1.4		4.4		5.4		8.1	
CV, %																
Repeatability	63.2		59.6		37.3		67.2		98.8		29.8		37.5		24.7	
Reproducibility	74.8		70.6		58.9		76.2		96.8		43.5		37.5		30.9	

[a]Counts by number of particles with hair (50 g/sample); duplicate samples at each spike level.
[b]Counts by number of positive spots (10 g/sample).
[c]Technical outliers by laboratory not included in statistical analysis (see text).
[d]Statistical outliers (Dixon test) by laboratory not included in statistical analysis.

2 times vol. of H_2O. Reserve stock test reagent. Place beaker of H_2O on hot plate stirrer, add stirring bar (ovoid 41 mm), and, with rapid stirring, add sufficient agar to H_2O to yield 2% agar soln (1.5 g agar/75 mL H_2O). Continue stirring, and heat to boil (watch for foam-over). Cover beaker with cover glass to prevent heat loss. When agar begins to foam, add reserved stock test reagent, pouring reagent down side of beaker to prevent agar from coming out of soln. Stir rapidly with heat ca 1 min.

44.B04 ***Determination***

Weigh 10 g corn meal into weighing boat from each well mixed subsample. Prep. appropriate amt of liq. test agar, (c). Cool boiled test agar by placing beaker of test agar into larger beaker of cold H_2O. Continually *stir* test agar and *maintain* temp. check until soln is 55°. Pour test agar into petri dish, ca 150 mL per dish. Immediately distribute monolayer of corn meal onto surface of test agar. This is accomplished by gently tapping weighing boat held so that corn meal flows over one side, not from corner, while tilting and moving boat above agar surface as corn meal flows. Let corn meal become wet with test agar and sink before adding another layer. Continue in this manner until entire 10 g sample has been added. Distribution time should be ca 1 min per 10 g corn meal sample. Best sepn of corn meal and excreta occurs while test agar is hot. Multiple samples can be added to resp. dishes, one at a time or a little of each sample to its resp. dish sequentially, until all of each sample has been distributed.

Let test agar gel (requires ≥20 min). Agar is gelled when no agar flows when dish is slightly tipped. (Caution: Take care not to disturb dispersed material in liq. test agar. If particles are moved, color concn around particles will be diffused and pos. spots will be missed.) When gelling is complete, check for pink spots, viewing plate against white background. Mark spots on lid of dish, using grease pencil. Mark lid and bottom of dish, using H_2O-proof marker, so that lid can always be placed in same position.

Incubate petri dish at 42° in H_2O bath 10 min. Submerge plate in H_2O bath just enough to cover agar level in dish. When incubating several dishes at one time, place plates in H_2O in pairs, staggering times so that reading delays are avoided and small, rapidly diffusing pink spots are not missed. Remove plate from H_2O bath after 10 min. Wipe inside lid to remove fog and hold lid so that bottom edge of lid is 2–3 mm above top edge of petri dish base while reading plate. Replace lid and repeat 10 min incubation 2 times, marking addnl pink spots on the petri dish lid after each period. Tally and record number of spots as fecal particles/10 g sample. Spots which appear and then are not seen on subsequent checks and spots which are seen on bottom of petri dish with corn meal are to be counted in tally.

44.B05 ***Positive Control for Feces and Test Agar Medium***

Scatter some ground known rodent feces on petri dish of liq. test agar in place of corn meal sample and continue with method. One control plate is needed for each batch of test agar prepd.

44.B06 ***Response***

Amt, intensity, and range of color (light pink to red-purple) observed will vary depending on size of fecal particles, species source, and diet of animal. Particles as small as 250 μm can be identified.

Results and Discussion

The results of the collaborative study are presented in Table 1. Results of Collaborator A for the official method and of Collaborator D for the proposed method were technical outliers and their data were not included in the statistical analysis. Collaborator A counted all particles recovered as fecal without checking for fecal characteristics, and Collaborator D doubled the amount of agar used in the test agar medium. Also not included in statistical data analysis were Dixon test outliers. The outliers in the official method were counts reported by Collaborator G for spike level 1; the outliers in the proposed method were counts by Collaborator H for spike levels 1 and 30 and by Collaborator I for the 1 particle level. Results from 1 collaborator were received after all other results were analyzed, and they are therefore not included in this report.

The counts by the official method were very low, 0–5, for all 4 spike levels (Table 1). The average recovery for all spike levels except the 1 particle level was 50.3% for the proposed method compared with 4.8% for the official method, i.e., 10.5 times greater. The greater precision of the proposed method

was demonstrated by lower coefficients of variation (CV) for both repeatability and reproducibility. Except for the 1 particle spike level, the repeatability and reproducibility of the official method were 1.8 times higher than those of the proposed method.

The experimental results for the proposed method yielded an average 50.7% recovery for all spike levels other than the 1 particle level. The collaborative study results are therefore consistent with experimental results. The 50% yield in the proposed method can be attributed in part to the spike material used. Not all of the particles of this spike material produced a positive response. Spike particles tested on the surface of the gelled agar without corn meal gave a positive response rate of 45–63%. The mechanics of the method introduced another source of apparent spike loss. Fecal particles can be trapped under corn meal when the product is applied to the test agar. Also, if corn meal is sprinkled too rapidly, not in a monolayer, particles may be covered and pushed to the bottom.

No background filth had been found during tests of the corn meal to be used for the collaborative study; however, some collaborators reported more than 1 particle in the 1 particle test portions. A possible explanation is that the large particles used for spiking broke into pieces during handling. In retrospect, it would have been better to have the collaborators add the control particles to the agar instead of mixing them in the test portions before shipping.

All collaborators felt that the proposed method was superior to the official method in that no toxic chemicals were required and the method was simple, easy to perform, and much more sensitive than the official method. Many collaborators stated that the number of positives they reported for the official method was based on particles picked out of the corn meal that was to be discarded, not on solvent separation.

With the present official method for rodent filth in corn meal, fecal particles are positively identified by the presence of rodent hair and a mucous coating. The proposed method introduces a different means of identifying mammalian feces and a more sensitive level of fecal detection.

Recommendation

The proposed method gives higher recoveries and better precision than the official method. It eliminates the use of toxic chemicals and is rapid to perform. It is recommended that the proposed method be adopted official first action and that study be continued using this method for other food products.

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References

(1) *Official Methods of Analysis* (1984) 14th Ed., AOAC, Arlington, VA, sec. **44.049**

(2) *Official Methods of Analysis* (1984) 14th Ed., AOAC, Arlington, VA, secs **44.181–44.184**

Improved Spray Reagent for Thin Layer Chromatographic Method for Detecting Uric Acid: Collaborative Study

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A collaborative study was conducted to validate the substitution of an improved single spray in the official AOAC thin layer chromatographic method for identifying uric acid (UA) from bird and insect excreta. The proposed reagent, which is a dilute aqueous solution of ferric chloride and potassium ferricyanide, requires neither a heating step nor a pH indicator. Its preparation time, specificity, and sensitivity to low levels (5–50 ng) of UA were compared with those of the official sprays. The improved spray took 1/5 as long to prepare as the official sprays. Neither the proposed spray nor the official sprays gave false positive reactions with compounds similar to UA. For bird and insect excreta samples, at the 95% confidence limits, the false negative rate was between 0 and 9.7% for the proposed spray and between 0.7 and 18.7% for the official sprays. Sensitivity results showed that the proportion positive for the proposed spray was significantly higher ($P<0.05$) than for the official sprays at the 15 ng UA level. The proposed changes have been adopted official first action.

The official AOAC thin layer chromatographic (TLC) method for identifying uric acid (UA) from bird and insect excreta (1) consists of spotting lithium carbonate extracts of suspect materials on a cellulose plate, developing the plate in methanol–butanol–water–acetic acid, observing it under shortwave (254 nm) UV light, spraying first with tribasic sodium phosphate (reagent A) and then with phosphotungstic acid (reagent B), and heating the plate to develop spot color. If all spotted materials have not responded, the plate is resprayed with reagent A. The method has certain disadvantages attributable to the spray reagents: A great deal of time is needed to prepare reagent B, which includes a 1 h reflux step; the plate must be spotted with a pH indicator, phenolphthalein, before the sprays are applied; a heating step is required after the sprays are applied; it is sometimes necessary to respray with reagent A for a total of 3 spray applications to see the color development clearly; and it is difficult to observe low levels of UA because of the resultant light blue color of the spots.

An improved procedure using a single spray was developed. The spray, which is a dilute form of a spray reagent (No. 111) listed by Krebs et al. (2) for the detection of compounds with reducing properties, is an aqueous solution of potassium ferricyanide and ferric chloride. Experimental studies have shown it to be superior to the present official sprays with respect to preparation time and response to low levels (5–50 ng) of UA. The greater sensitivity is due to the deeper blue color of its reaction product with UA as compared with the light blue color of the product formed when UA reacts with the official sprays. These studies have also shown that the proposed reagent is as effective as the official sprays in not giving false positive reactions in this procedure.

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The recommendations of the Associate Referee were approved by the General Referee and the Committee on Microbiology and were adopted by the Association. See the General Referee and Committee reports, *J. Assoc. Off. Anal. Chem.* (1986) 69, March issue.
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Experimental

We found that the proposed spray reacted with the cellulose of the plates to give a blue background color, which could mask the color of the UA spots. Even brief heating of the plate at low temperature (about 50°C) and/or spraying excessively with the proposed reagent resulted in accelerated development of this background color, with no significant increase in the intensity of the color of the UA spots. We minimized this problem by eliminating the heating step completely and spraying the plates lightly and evenly only until the blue UA spots clearly appeared. Marking the spots immediately on appearance further ensured that they would not be obscured by background color.

In testing the proposed spray for specificity, Analtech® (Analtech, Inc., Newark, DE 19711) plates were spotted with the official UA standard working solution, Li_2CO_3 solution extracts of bird and insect excreta, and Li_2CO_3 solutions of 5 compounds chemically related to UA (caffeine, hypoxanthine, theobromine, theophylline, and xanthine). There were no false positive reactions. As another part of this testing, a sprayed plate was examined with longwave (365 nm) UV light. The UA standard and bird and insect excreta spots showed a weak, dark bluish purple fluorescence, slightly more intense with transmitted than with reflected light. None of the related compounds fluoresced. Based on these results, longwave UV examination was included in this study to evaluate its usefulness as a possible confirmatory test for UA.

With the proposed spray, as little as 10 ng UA (and occasionally 5 ng) consistently reacted, whereas with the official method, 25 ng UA (and occasionally 20 ng) consistently reacted. The 2 component solutions of the proposed spray were found to be stable for about 2 weeks when they were refrigerated and when solution A was protected from light.

Collaborative Study

Ten collaborators compared the reagent preparation time, specificity, and sensitivity to low levels of UA of the single proposed spray with those of the 2 official sprays.

Reagent Preparation Time

Collaborators prepared all reagents. They were asked to report the total preparation time for both official sprays and for the 2 component solutions of the proposed spray.

Specificity

The Associate Referee (AR) furnished each collaborator with 9 preweighed samples of unknowns in powdered form. Four of the unknowns, which contained UA, and the levels tested were bird excreta (Japanese quail, *Coturnix coturnix japonica*) at 0.2 and 0.1 μg/μL (unknowns E and I, respectively); beetle adult and larval excreta (yellow meal worm, *Tenebrio molitor*) at 2 μg/μL (unknown G); and moth larval excreta (Indianmeal moth, *Plodia interpunctella*) at 2 μg/μL (unknown L). The concentrations of these 4 unknowns were set at 1/2 the amounts used in the original study by Thrasher and Abadie (3) because we found that these levels yielded

Table 1. Preparation times for official and proposed spray reagents in uric acid thin layer chromatographic method

Coll.	Time, min	
	Official sprays[a]	Proposed spray[b]
A	150	30
B	180	30
C	150	30
D	100	20
E	300	30
F	150	15
G	105	10
H	150	30
I	120	30
J	120	20
Mean	152.5	24.5
Range	100–300	10–30

[a]Combined time to prepare spray reagents A and B.
[b]Combined time to prepare solutions A and B.

easily detectable spots of appropriate size with the proposed spray reagent. Japanese quail excreta were used as unknown samples of bird excreta because of their availability. They gave the same response with the proposed and official sprays as pigeon and chicken excreta, which are more commonly found as food contaminants. The other 5 samples were compounds chemically related to UA. These, and the levels tested, were: theophylline, 1.6 μg/μL (unknown F); xanthine, 1.4 μg/μL (H); caffeine, 2.0 μg/μL (J); theobromine, 1.6 μg/μL (K); and hypoxanthine, 0.6 μg/μL (M).

Collaborators prepared spotting solutions of the UA standard and unknowns, treating the unknowns much like "other suspect material" in the official method. The UA standard was spotted at 4 levels: 0.100 μg/μL (unknown A); 0.050 μg/μL (B); 0.025 μg/μL (C); and 0.010 μg/μL (D). They spotted 2 plates with 1 μL of each unknown and the 4 dilutions of UA standard in the following format:

```
A     D        B     A           C        A
•  •  •  •  •  •  •  •  •  •  •  •  •  •  •  •
   E     F  G     H     I  J  K     L  M
```

Shortwave UV observation of plates for quenching before spraying was made optional because of potential problems associated with reduced transmission of this wavelength through some types of UV lamp filters with prolonged use (i.e., solarization). The collaborators used the official sprays and color development procedure for 1 plate and the proposed spray and color development procedure for the other. They observed the latter plate with longwave UV light. They were asked to report all spots reacting positively on the plate treated with the official sprays, regardless of when they appeared in the color development procedure. For the plate sprayed with the proposed spray, they were asked to report all spots that appeared after spraying once, all additional spots that appeared after respraying, and all fluorescent spots observed with longwave UV.

Sensitivity

For the principal sensitivity testing, collaborators performed the official and the proposed color development procedures, using the spray reagents they prepared on plates that had been previously spotted with low levels of UA standard and developed by the Associate Referee. Preliminary stability testing was done to determine whether the UA spots would remain stable for the period between spotting/developing the plates and completion of testing by the collaborators. Over a period of 47 days, the Associate Referee tested the proposed spray on several plates (1 per testing day), each of which had been spotted with duplicate low levels (5–40 ng) of UA standard, developed, dried, individually covered with glass and aluminum foil, and stored at room temperature. The authors detected 5–10 ng UA even as late as day 32. Because the spots are stable for at least a month, it was possible to send collaborators prespotted, predeveloped plates without noticeable decomposition of these spots occurring before collaborators completed the sensitivity testing.

All the plates sent to the collaborators were spotted in the same overall pattern. The left and right halves of the plates were spotted with 1 μL of each of 8 levels of UA standard (50, 40, 30, 25, 20, 15, 10, and 5 ng). Each half was spotted in a mixed subpattern rather than in order of concentration, and the subpatterns on the 2 halves of each plate differed from each other. Collaborators were not informed of the UA concentrations spotted, the presence of duplicates, or the spotting pattern used. They were requested to report all positive reactions in the same manner as that requested for reporting specificity test results. This time, however, longwave UV observation was omitted. The 4 UA standard dilutions that the collaborators prepared and spotted as reference standards on the plates used for specificity testing also served as an additional test for sensitivity. Collaborators were asked if they had a preference for either the official sprays or the proposed spray, and if so, were requested to state the reason(s) for the preference.

Excrement (Bird and Insect) on Food and Containers

Thin Layer Chromatographic Method for Uric Acid

First Action

(Applicable to suspect material not suitable for detn by **44.185** and/or to confirmation of **44.185** when adequate material is available.)

44.B07 ***Apparatus and Reagents***

(a) *Thin layer cellulose plates.—See* **44.176**. E. Merck cellulose plates, 0.10 mm, EM No. 5757-7 (EM Science, Cherry Hill, NJ 08034) have also been found satisfactory.

(b) *Cellulose powder.—See* **44.003(g)**.

(c) *Detection spray.—(1) Soln A.*—1% $K_3Fe(CN)_6$. *(2) Soln B.*—2% $FeCl_3$ (calcd as anhyd.). Refrigerate both solns. Protect soln A from light. Solns are stable ca 2 weeks. *(3) Spray reagent.*—To 18 mL H_2O, add 1 mL each of solns A and B; mix. Prep. *immediately* before use.

(d) *Developing solvent.*—*n*-BuOH–MeOH–H_2O (4 + 4 + 3). Measure vols sep. and mix well to form stable single phase. To 30 mL of this soln, add 1 mL HOAc; mix well. Prep. fresh daily.

(e) *Dye mixture.*—Dissolve 16 mg amaranth (formerly FD&C Red No. 2) and 32 mg FD&C Yellow No. 6 in 50 mL H_2O; mix well.

(f) *Lithium carbonate soln.*—1 mg/mL.

(g) *Uric acid std soln.—(1) Stock soln.*—1 mg/mL. Dry 105 mg uric acid in 100° oven overnight and cool to room temp. in desiccator. Accurately weigh 60 mg Li_2CO_3 and transfer to 100 mL vol. flask. Accurately weigh 100 mg cool uric acid and transfer quant. to the 100 mL flask with ca 50 mL H_2O. Place in 60° H_2O bath and agitate until soln clears. Cool immediately under tap H_2O to room temp. and dil. to vol. with H_2O. For short term use (<3 days), store in refrigerator; for extended use, place portions in small containers and store hard-frozen. *(2) Working soln.*—100 μg/mL. Pipet 10 mL stock soln into 100 mL vol. flask and dil. to vol. with H_2O. Prep. fresh daily.

44.B08 ***Preparation of Sample***

(a) *Insect excreta.*—Transfer material to small test tube, crush with glass rod, and add 0.05–0.10 mL Li_2CO_3 soln, **(f)**. Let soak ca 10 min and centrf. Obtain clear supernate and proceed as in **44.188**.

(b) *Paper bags or cartons.*—Cut 5–6 mm diam. portion from suspect area. Cut another 5–6 mm portion from nearby unstained area as neg. control. Place individually in small test tubes. Add ca

Table 2. Uric acid thin layer chromatographic specificity tests for unknowns containing uric acid (bird and insect excreta)[a]

	Bird excreta (0.2 μg)[b]		Bird excreta (0.1 μg)		Beetle excreta (2.0 μg)		Moth excreta (2.0 μg)	
Coll.	Off.	Prop.	Off.	Prop.	Off.	Prop.	Off.	Prop.
A	+	+	+	+	+	+	+	+
B	+	+[c]	−	+[c]	−	+	+	+[c]
C	+	+[c]	+	+[c]	+	+[c]	+	+[c]
D	+	+[c]	+	+[c]	+	+[c]	+	+[c]
E	+	+[c]	+	+[c]	+	+[c]	+	+[c]
F	+	+	+	+	+	+	+	+
G	+	+[c]	+	+	+	+	+	+[c]
H	+	+	+	+	+	+	+	+
I[d]	(−)	(+)	(+)	(+)	(+)	(+[c])	(+)	(+[c])
J	+	+	+	+	+	+	+	+

[a]Off., official sprays; prop., proposed spray; +, positive; −, negative.
[b]Values in parentheses are weight of excreta extracted per spot.
[c]Spot fluoresced with longwave (365 nm) UV light.
[d]Collaborator used silica gel plates; therefore, these results were not included in the statistical analysis.

0.1 mL Li_2CO_3 soln, (f), to each tube; agitate with small stirring rod. Let soak ca 10 min and proceed as in **44.188**.

(c) *Other suspect material.*—Transfer small portion to test tube, add ca 0.1 mL Li_2CO_3 soln, (f), and stir with glass rod. Let soak ca 10 min; centrf. Obtain clear supernate and proceed as in **44.188**.

44.B09 ***Determination***

(a) *Spotting of plates.*—Place coated plate on heated metal slab reading ca 87° on surface thermometer or 70° on 3 in. (76 mm) immersion thermometer inserted through hole in stopper until tip touches bottom of 250 mL conical flask contg 125 mL glycerol. *Caution:* Plates tend to crack, particularly prescored plates, unless heated evenly. Place infrared lamp or forced hot air source (e.g., hair dryer) above plate to speed drying of spots. Spot 1 μL uric acid working std soln, (g)(2), at each edge and at center of plate ca 15–20 mm from bottom. Spot 1 μL dye mixt., (e), to side of each working std spot. These dyes serve as visual markers during development, with R_f for amaranth at 0.38–0.40; uric acid, 0.41–0.43; and Yellow No. 6, 0.65, using Analtech plate and sandwich chamber. Merck plates have lower R_f values, with R_f for amaranth approx. equal to that of uric acid. Spot samples and neg. controls along same line at ≥10 mm intervals. Keep spots at min. size by drying well between successive small addns.

(b) *Development of plates.*—Scribe horizontal line, ca 1 mm wide, across plate exactly 10 cm above origin, completely removing cellulose layer. Develop to this line in conventional satd tank without pre-equilibration or, alternatively, form sandwich chamber with uncoated plate [See **44.175(b)** and **44.177(c)** and (f)] and develop. Dry plate on heated metal slab or in forced draft oven ca 5 min at 75–80°.

(c) *Examination with UV light.*—Observe plate under shortwave (254 nm) UV light in darkened room, marking each quenching (dark) spot with penciled dots at top, bottom, left, and right edges. Shortwave lamps in fluorescent tube style have integral filters with transmission characteristics that change with use. Some UV viewing cabinets have label attached calling attention to this fall-off of transmittance of 254 nm. High levels of uric acid should appear as dark spots at $R_f = 0.40 \pm 0.05$, depending on conditions of development.

(d) *Color development.*—Spray plate evenly in hood, concentrating on horizontal zone between upper (yellow) dye spots and ca 2 cm below lower (red) dye spots, only until blue uric acid spots clearly appear at R_f stated in (c). Immediately outline spots with soft (No. 1) pencil, marking weakest spots first. Continue spraying *only until background begins to darken.* Immediately outline any addnl spots which appear (again, weakest ones first). (*Caution:* Excessive spraying accelerates plate darkening.)

Results

The comparative preparation times for the official and proposed sprays are presented in Table 1. The proposed spray took about 1/5 as long to prepare as the official sprays.

The results of specificity tests for those samples of unknowns that should have responded positively are presented in Table 2. The proposed spray performed successfully 100% of the time for all materials tested. The official sprays performed successfully 100% of the time for moth excreta and the higher level of bird excreta; they performed successfully 89% of the time for beetle excreta and the lower level of bird excreta. Based on statistical analysis of the data, one may be 95% confident that the false negative rate is between 0 and 9.7% for the proposed spray and between 0.7 and 18.7% for the official sprays. One collaborator used silica gel plates because no cellulose plates were available. Because the method specifies only cellulose plates, the results obtained by this collaborator were identified as a technical outlier due to a significant deviation from the method and were not included in the statistical analysis of the results.

The results of the specificity tests for compounds similar to UA showed negative results for all samples tested with both the proposed and the official sprays. One collaborator reported a positive (blue) color reaction for xanthine with the proposed spray; however, the R_f of the spot was ca 0.59, vs 0.43–0.45 for UA, and therefore it is a negative result. Based on statistical analysis, one may be 95% confident that the false positive rate is between 0 and 7.9%.

Results of the sensitivity tests on plates that were prespotted and predeveloped by the Associate Referee are presented in Table 3. For statistical analysis, if a particular level of UA was reported as positive on at least half of the plate, it was considered positive. Based on this analysis, the proportion of positive reactions for the proposed spray and the official sprays did not differ significantly ($P>0.10$) for 6 levels of UA (50, 40, 30, 20, 10, and 5 ng). However, for the 25 and 15 ng levels, the proportion positive for the proposed spray was significantly higher than for the official sprays ($P<0.05$) (see *Discussion*).

Results of the sensitivity tests on plates that were completely processed by collaborators are presented in Table 4. Based on statistical analysis, one may be 95% confident that the false negative rate is between 3.1 and 25.9% for the proposed spray and between 16.4 and 48.1% for the official sprays. There was no significant difference ($P>0.25$) between the proposed and official sprays in terms of proportion positive.

The results of the longwave UV examination of collaborator-prepared plates after spraying with the proposed spray are included in Tables 2 and 4. These results were not statistically analyzed. No false positives were reported. Only 1 collaborator reported fluorescence for all 8 spots that were

Table 3. Uric acid thin layer chromatographic sensitivity tests on prespotted, predeveloped plates[a]

	Weight of uric acid per spot, ng[b]																													
	50				40				30				25				20				15				10				5			
	Off.		Prop.		Off.		Prop.		Off.		Prop.		Off.		Prop.		Off.		Prop.		Off.		Prop.		Off.		Prop.		Off.		Prop.	
Coll.	L	R	L	R	L	R	L	R	L	R	L	R	L	R	L	R	L	R	L	R	L	R	L	R	L	R	L	R	L	R	L	R
A	+	+	+	+	+	+	+	+	−	+	−	+	+	−	+	+	−	−	−	+	−	−	−	+	−	−	−	−	−	−	−	−
B	−	−	+	+	−	−	+	+	−	−	+	+	−	−	+	+	−	−	+	+	−	−	+	+	−	−	−	−	−	−	−	−
C	+	+	+	+	+	−	+	+	−	+	+	+	−	−	+	+	+	−	+	+	−	−	+	+	−	−	+	−	−	−	−	−
D	+	+	+	+	+	+	+	+	+	+	+	+	−	−	+	+	−	+	+	+	−	−	−	+	−	−	−	−	−	−	−	−
E	+	+	+	+	+	+	+	+	−	+	+	+	−	−	+	+	+	+	+	+	−	−	+	−	−	−	−	+	−	−	−	−
F	+	+	+	+	+	+	+	+	−	+	+	+	−	−	+	+	−	−	+	+	−	−	+	+	−	−	+	−	−	−	−	−
G	+	+	+	+	+	−	+	+	−	+	+	+	+	+	+	+	+	+	+	+	−	−	−	+	+	−	+	−	−	−	−	−
H	+	−	+	+	+	+	+	+	−	−	+	+	+	−	+	−	+	+	+	+	−	−	+	+	−	−	−	−	+	−	−	−
I	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	−	+	+	+	+	−	+	+	−	−	−	−
J	+	−	+	+	−	−	+	+	−	−	+	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	+

[a]Off., official sprays; prop., proposed spray; L, left half of plate; R, right half of plate; +, positive; −, negative.
[b]Spotting pattern: L 30, 5, 40, 20, 25, 10, 50, 15; R 10, 40, 5, 30, 20, 50, 15, 25.

Table 4. Uric acid thin layer chromatographic sensitivity tests on plates processed by collaborators[a]

	Weight of uric acid per spot, ng							
	100		50		25		10	
Coll.	Off.	Prop.	Off.	Prop.	Off.	Prop.	Off.	Prop.
A	+	+	+	+	−	+	−	−
B	+	+[c]	−	+	−	−	−	−
C	+	+[c]	+	+[c]	+	+[c]	−	+
D	+	+[c]	+	+[c]	+	+[c]	+	+[c]
E	+	+[c]	+	+[c]	+	+	−	+
F	+	+	+	+	−	+	−	−
G	+	+[c]	+	+	+	+	−	+
H	+	+	+	+	+	+	+	+
I[b]	(+)	(+)	(+)	(+)	(−)	(+)	(−)	(−)
J	+	+	+	+	−	+	+	+

[a]Off., official sprays; prop., proposed spray; +, positive; −, negative.
[b]Collaborator used silica gel plates; therefore, these results were not included in the statistical analysis.
[c]Spot fluoresced with longwave (365 nm) UV light.

expected to give positive results (i.e., UA standards and bird and insect excreta). Another collaborator reported positives for all but the lowest level of UA standard (10 ng). Another reported positives for all but the 2 lowest levels of UA standard (25 and 10 ng). Two others reported positives for only the highest level of UA standard (100 ng), but did not report all possible positives for bird and insect excreta. Three collaborators reported all 8 expected positives as negative.

With respect to detection spray preference, all 6 collaborators who responded preferred the proposed spray over the official sprays. Their collective reasons included the brevity and ease of its preparation, the earlier appearance and deeper color of spots (despite increased plate background color), omission of the heating step, and the desirability of 1 spray reagent rather than 2 in TLC methods.

Discussion

The results generally confirmed those of the precollaborative experimental study. Considerable time, about 2 h, is saved in preparing the detection spray. The method is also simplified in that the number of sprays and spray applications is reduced, the heating step is omitted, and a pH indicator is not needed.

The positive color reaction reported by 1 collaborator for xanthine was unexpected and cannot be explained. The resulting blue spot could not be mistakenly identified as UA because of the clearly different R_f (ca 0.59 vs 0.43–0.45 for UA), but users of the method should be aware of a possible reaction with xanthine or 1 of its decomposition products.

Several collaborators indicated that longwave UV observation of plates after spraying with the proposed reagent was of little or no value as a confirmatory test because they saw either very weak fluorescent spots or none at all. The results suggest a great deal of individual difference in the ability to detect weak fluorescence; therefore, longwave UV observation of sprayed plates will not be included in the method.

The proposed spray is significantly more sensitive than the official reagents at the 15 ng level ($P<0.05$). However, the greater number of negatives at the 25 ng UA level with the official sprays can be explained as a "position effect." One of the two 25 ng spots occupied the position nearest the right edge of the plate. This spot probably failed to react with the official sprays simply because the amount applied at the edge of the plate was sometimes insufficient. Plates treated with the proposed spray and spotted in the same pattern did not show this effect. Similar results were seen on the left edge of the plate, where 1 of the 30 ng spots was located. The difference between the proposed and official sprays was not significant in this instance, but most (8/11) of the negative results for the official sprays occurred there.

Such results suggest that the proposed spray is more reliable than the official sprays. That this is not simply a matter of increased sensitivity is indicated by the false negatives with the official sprays at the 50 and 40 ng levels of UA, the beetle excreta, and the lower level of bird excreta.

One collaborator, who used a tank to develop Analtech plates, reported R_f values for UA slightly below those of the lower (red) dye spots; i.e., the R_f of the UA spots was within expected limits, but the R_f of the dye spots was clearly higher than that of the UA spots. This may have been caused by not saturating the tank before inserting the plates. This collaborator may have misinterpreted the instructions in the plate

development section of the method. We are revising these instructions to read "conventional saturated tank" instead of "conventional tank." One can avoid this problem by using a sandwich chamber. However, as a further precaution against not spraying low enough on the plate to cover UA spots when they are likely to have a relatively low R_f compared with that of the red dye spots (e.g., when Merck plates are used), the spraying instructions, ". . . spray plate evenly, concentrating on horizontal zone between upper (yellow) and lower (red) dye spots," have been modified to read, ". . . spray plate evenly, concentrating on horizontal zone between upper (yellow) dye spots and ca 2 cm below lower (red) dye spots."

Recommendations

On the basis of speed and simplicity of spray reagent preparation, improved sensitivity, and the complete absence of false positives, it is recommended that the proposed detection spray and color development procedure replace those of the official first action method, **44.186–44.188**. It is further recommended that the following editorial changes be made in **44.186–44.188**: (a) inclusion of alternative TLC plates; (b) inclusion of negative control sample(s); (c) inclusion of caution statement relevant to plate cracking in spotting procedure; and (d) clarification of plate development procedure.

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HAZARDOUS SUBSTANCES

Gas Chromatographic-Thermal Energy Analysis Method for *N*-Nitrosodibutylamine in Latex Infant Pacifiers: Collaborative Study

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Each of 5 collaborating laboratories determined volatile *N*-nitrosamines in 3 blind quadruplicate sets of latex rubber infant pacifier samples, using a gas chromatographic-thermal energy analysis (GC-TEA) method. Volatile *N*-nitrosamines are extracted from cut-up pacifier nipples with CH_2Cl_2. The extract is concentrated and subjected to high temperature purge and trap, and the nitrosamines are eluted from the trap and determined by GC-TEA. *N*-Nitrosodibutylamine (NDBA) was the only nitrosamine found in sufficient concentration to allow analysis. NDBA concentrations of the 3 sets of samples were 82.6, 21.0, and 7.12 ng/g rubber. The repeatability relative standard deviations ranged from 7.46 to 24.0% and the reproducibility relative standard deviations from 7.46 to 29.2%. The minimum detectable level of NDBA by this method is 3.6 ng/g rubber. The method has been adopted official first action.

N-Nitrosamines are present in rubber products vulcanized with accelerators and stabilizers that were derived from dialkylamines (1). Raw polymers, compounded uncured elastomers, and cured rubber parts containing dialkylamine compounds also emit the corresponding dialkylnitrosamines when they are heated (2). The rubber tire industry has long been known to have a higher than average rate of cancer incidence among its workers, as evidenced by epidemiological studies (3, 4). Recent studies reported the presence of volatile *N*-nitrosamines in the air of rubber factories (5, 6). The origin of the *N*-nitrosamines in the factory air was traced to the accelerators and stabilizers used in the vulcanization process (1, 2).

In tests on laboratory animals, 85% of 209 *N*-nitroso compounds and 92% of 86 nitrosamines tested have been shown to be carcinogenic (7). As an example, *N*-nitrosodiethylamine (NDEA) has been tested and shown to induce cancer in 20 species of animals (8).

Recent findings by Preussmann et al. (9) indicate that the rubber nipples on baby bottles and pacifiers contain volatile *N*-nitrosamines that can be extracted into an aqueous saliva simulant. It is possible for an infant to ingest these toxic chemicals when feeding from a baby bottle or using a pacifier. Billedeau et al. (10) recently examined pacifiers of all brands sold in the United States for volatile *N*-nitrosamines and found that *N*-nitrosodibutylamine was the principal nitrosamine, at levels up to 332 ppb. The method used by Billedeau et al. was a modification of the procedure of Havery and Fazio (11) together with the procedure of Rounbehler et al. (12). On January 1, 1984, the U.S. Consumer Product Safety Commission established an action level of 60 ppb total volatile *N*-nitrosamines in latex infant pacifiers. The method of Billedeau et al. was studied collaboratively to determine its suitability to support the current 60 ppb volatile *N*-nitrosamine action level or concentrations as low as 10 ppb in the event the action level is reduced to that extent in the future. The method was selected because it is more time-efficient and therefore more cost-effective to perform than other procedures.

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The recommendation of the Associate Referee, H. C. Thompson, Jr, was approved by the General Referee and the Committee on Hazardous Substances in Water and the Environment and was adopted by the Association. See the General Referee and Committee reports, *J. Assoc. Off. Anal. Chem.* (1986) **69**, March issue.

Collaborative Study

The method was submitted to 5 collaborating laboratories along with 3 blind quadruplicate sets of pacifier nipple samples, *N*-nitrosodipropylamine (NDPA) internal standard, and an external standard containing *N*-nitrosodimethylamine (NDMA), NDEA, NDPA, *N*-nitrosodibutylamine (NDBA), *N*-nitrosopiperidine (NPIP), *N*-nitrosopyrrolidine (NPYR), and *N*-nitrosomorpholine (NMOR). ThermoSorb/N™ cartridges from the same lot and Tygon™ connectors were supplied to each laboratory. Collaborators were instructed to use the internal standard technique, with NDPA as internal standard, and to report concentrations of nitrosamines detected in ng/g (ppb).

Preparation of Collaborative Samples

Pacifiers used for preparation of a composite were selected from a single lot because of the high degree of variability in nitrosamine composition among lots. Sufficient pacifier nipples to form a 200 g composite were excised from their plastic or rubber base and cut into 1–2 mm chips with dichloromethane-rinsed stainless steel forceps and scissors. The composite was sticky after it was cut, which made homogenization very difficult. To break up the large clumps of rubber, the sample was placed in a 2-quart stainless steel Waring blender jar and liquid nitrogen was poured into the jar to cover the rubber chips. The excess liquid nitrogen was then decanted into a waste Dewar flask (insulated gloves were used to handle the extremely cold metal jar). The frozen rubber chips were homogenized by blending at approximately 40% maximum speed for 2 min. The homogenized rubber chips were then poured into a 1-gallon amber glass sample jar with an aluminum foil-lined screw cap. (Note: Care must be taken to avoid addition of any small balls of powdered rubber which might be formed in the blending process.) Homogeneity was evaluated by analysis of 4 replicates from each composite. Coefficients of variation of the 3 composites ranged from 2.4% for A (82 ng/g) to 6.2% for C (7 ng/g). Each composite was then stored in a freezer at −20°C until needed for distribution to collaborators.

N-Nitrosodibutylamine in Latex Infant Pacifiers

Gas Chromatographic Method

First Action

5.B01 ***Principle***

Volatile *N*-nitrosamines are extd from cut-up latex pacifier nipples with CH_2Cl_2. Ext is concd and subjected to high temp. purge and

Table 1. Collaborative results[a] for determination of NDBA in latex rubber infant pacifiers

	Composite A sample set[b]				Composite B sample set				Composite C sample set			
Lab.	1	5	8	11	2	4	7	10	3	6	9	12
1	91.4	80.5	88.6	75.6	17.4	17.4	19.7	20.8	12.2	7.2	7.4	11.5
2	74.3	91.7	85.5	76.0	22.8	19.1	19.5	20.3	8.4	5.1	5.3	5.5
3	168	125	98.8	109	27.0	18.3	21.6	24.7	7.0	7.9	5.5	5.9
4	77.9	88.1	81.0	76 2	16.6	30.6	25.8	23.4	5.8	5.8	9.7	7.8
5	86.0	84.8	81.5	82 8	19.7	19.8	18.5	17.8	6.5	6.8	6.0	5.1

[a]Units are ng NDBA/g rubber.
[b]Blind replicate numbers.

trap, and *N*-nitrosamines are eluted from trap and detd by gas chromatgy with thermal energy analysis.

5.B02 *Reagents*

Use all glass-distd solvs (Burdick & Jackson Laboratories, Inc., or equiv.).

(a) *N-Nitrosamine stock std solns.*—(*1*) *External stock std soln.*—10 μg/mL each of NDMA (*N*-nitrosodimethylamine), NDEA (*N*-nitrosodiethylamine), NDPA (*N*-nitrosodipropylamine), NDBA (*N*-nitrosodibutylamine), NPIP (*N*-nitrosopiperidine), NPYR (*N*-nitrosopyrrolidine), and NMOR (*N*-nitrosomorpholine) in alcohol. (*2*) *Internal stock std soln.*—10 μg NDPA/mL alcohol.

Caution: Volatile *N*-nitrosamines are extremely hazardous compds. Carry out all manipulations involving handling neat liqs or solns in adequately ventilated and filtered fume hood or glove box.

(b) *Mineral oil.*—White, lightwt Saybolt viscosity 125/135 (No. 6358, Mallinckrodt Chemical Works).

(c) *Nitrosation inhibitor.*—10 mg α-tocopherol/mL mineral oil.

(d) *Keeper solns.*—(*1*) *For K-D evaporation.*—80 mg mineral oil/mL CH_2Cl_2. (*2*) *For N evaporation.*—20 mg mineral oil/mL isooctane.

5.B03 *Apparatus*

(a) *ThermoSorb/N™ cartridges.*—Use as received for quant. trapping of volatile *N*-nitrosamines (Thermedics, Inc., Div. of Thermo Electron Corp., Woburn, MA 01801).

(b) *Variable temperature oil bath.*—Thermostatically controlled, capable of operating at 150 ± 3° and of moving vertically with aid of laboratory jack (The Lab Apparatus Co., PO Box 42070, Cleveland, OH 44142).

(c) *Soxhlet extraction apparatus.*—(Kimble Glass Co.). Allihn condenser with 34/45 ℨ joint. Extn tube with 34/45 ℨ upper joint and 24/40 ℨ lower joint. Extn thimble, 25 × 85 mm borosilicate glass fitted with coarse porosity frit.

(d) *Kuderna-Danish evaporative concentrator.*—(Kontes Glass Co.). 3-ball Snyder column with 24/40 ℨ joints, 250 mL flask with 24/40 ℨ joint and 19/22 ℨ lower joint, and 4 mL graduated concentrator tube with 19/22 ℨ joint.

(e) *Gas chromatograph.*—Hewlett-Packard Model 5710A, or equiv., equipped with 6 ft × 4 mm id glass column packed with 10% Carbowax 20M/2% KOH on 80–100 mesh Chromosorb WAW (No. 1-1805, Supelco). Condition column overnight at 215°. Operate at temp. program mode from 150 to 190° at 4°/min. Injection port temp. 250°. Carrier gas prepurified Ar at flow rate 40 mL/min. Interface GC app. to thermal energy analyzer, (f), via ⅛ in. od stainless steel tube connected to Swagelok fittings and operate at 170°.

(f) *Thermal energy analyzer.*—Model 502, Thermo Electron Corp., or equiv. Operate pyrolysis chamber at 500° in GC mode. O flow to ozonator, 10 mL/min. Keep cold trap at −150° using liq. N/2-methylbutane slush bath. Pressure of reaction chamber, ca 0.9 torr. Record TEA detector response on Hewlett-Packard 3380 integrator.

(g) *Purge and trap apparatus.*—Fig. **5:B1** contains following parts: (*1*) Ar gas cylinder and gauge (Air Products Specialty Gas, Tamaqua, PA 18252); (*2*) metering valve; (*3*) purge gas manifold, 4-position; (*4*) Nalgene needle valve type CPE (No. 6400-0125, Nalge Co., Rochester, NY 14602); (*5*) 18/7 g-g outer joints with pinch clamps (No. 772398, Wheaton Scientific, Millville, NJ 08332); (*6*) impingers, 50 mL graduated glass tubes with 24/40 ℨ clear-seal, grease-free joints, 18/7 g-g ball joints, and 1 mm id nozzle ca 5 mm above bottom of impinger (No. 753463, Wheaton Scientific); (*7*) variable scale flow-check, calibrated for purge rate in mL Ar/min (No. 7083, Alltech Associates, Inc.). Bubble meter for measuring gas flow rates for GC may be substituted.

Note: Do not use any rubber tubing, gaskets, O-rings, or other items made of rubber in any part of this method.

5.B04 *Description and Use of Purge and Trap Apparatus*

App. shown in Fig. **5:B1** is designed for high temp. purging and trapping of 7 volatile *N*-nitrosamines from concd sample ext/mineral oil mixt. on 4 samples simultaneously. Cylinder contg prepurified Ar gas equipped with high pressure regulator is used to supply 20 psig to flow-metering valve which regulates final purge flow thru samples. Gas stream is diverted into tubular stainless steel mainfold, 250 × 20 mm od, contg 4 exit tubes spaced 50 mm apart and measuring 40 × 10 mm od. Each of these tubes is coupled using ⅜ in. Tygon tubing to Nalgene needle valves which serve dual purposes: as shut-off valve when less than 4 samples are analyzed; and for making minor adjustments in purge rate due to slight differences in flow characteristics of impinger and cartridges. An 18/7 g-g outer spherical joint is attached to Nalgene valve to permit quick, gas-tight connection to 18/7 g-g ball joint on impinger inlet, using appropriate pinch clamp. As shown in Fig. **5:B2**, impingers are assembled by inserting glass nozzle (1 mm id orifice) into sample mixt. and coupling 24/40 ℨ grease-free male and female joints together to form leak-free seal. Once sealed, Ar gas is allowed to purge thru sample mixt., thru outlet tube of impinger (see Fig. **5:B2**). Tygon tubing is used to connect impinger outlet tube to inlet side marked "AIR IN" of cartridge, which is std male Luer connector. Purged volatile *N*-nitrosamines are then collected on sorbent contained in cartridge with Ar effluent exiting from female Luer connector. Flow rate of Ar is measured directly from cartridge with variable scale flow meter which has been previously calibrated for flow rate of Ar gas (mL/min). Bubble meter can be substituted for variable scale flow meter. Temp. of sample mixt. during purge is controlled by immersing impinger up to sample vol. mark (ca 25 mL line) in

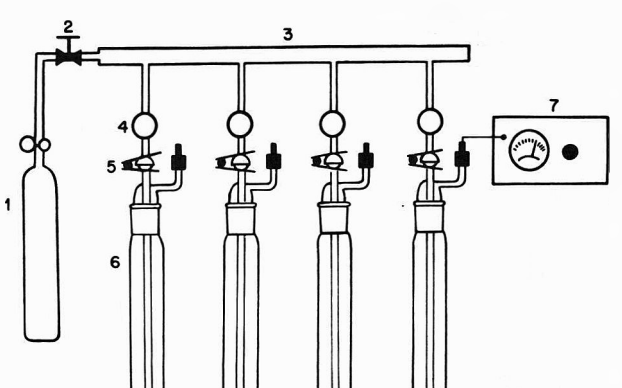

FIG. 5:B1—Diagram of purge and trap apparatus equipped with 4 impinger tubes

Table 2. Statistical summary of data (ppb) from collaborative study of method for NDBA in latex rubber infant pacifiers

Statistic	Composite A[a]		Composite B	Composite C
	I	II		
Mean	82.6	91.1	21.0	7.12
Repeatability S_o	6.16	14.7	3.31	1.71
RSD_o (%)	7.46	16.1	15.7	24.0
Reproducibility S_x	6.16	23.0	3.72	2.08
RSD_x (%)	7.46	25.2	17.7	29.2
Outlier		Lab. 3[b]		

[a]I = computed without data from Lab. 3; II = computed with data from Lab. 3.
[b]Determined to be an outlier by Cochran test.

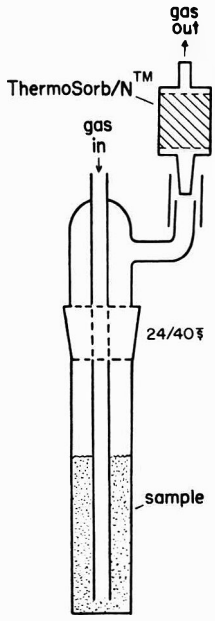

FIG. 5:B2—Close-up diagram of impinger tube fitted with ThermoSorb/N cartridge

thermostatically controlled oil bath capable of operating isothermally up to 150°. Gas manifold, as well as each impinger, is secured by clamps to support grid; therefore, oil bath is moved vertically in and out of position for high temp. purge.

5.B05 *Extraction and Cleanup of Pacifier Samples*

Accurately weigh 5 g from each sample into 250 mL r-b flask and add 100 mL CH_2Cl_2. Dil. internal stock std soln to 50 ng/mL with CH_2Cl_2 and spike contents of flask with 2 mL dild std. Seal flask and let contents stand overnight (16–21 h) at ambient temp.

Transfer ext and rubber pieces to glass extn thimble fitted with coarse porosity glass frit in Soxhlet extn app. Rinse 250 mL r-b flask with 25 mL CH_2Cl_2 and transfer rinse to Soxhlet app. Ext rubber pieces for 1 h in app. at rate of 8 cycles/h.

Let cool and transfer CH_2Cl_2 ext to 250 mL K-D evaporator. Rinse extn flask with two 10 mL portions of CH_2Cl_2 and combine rinses with 125 mL ext. Add 1 mL keeper soln *1* and 2 or 3 boiling chips (Boileezers, Fisher Scientific Co.) to ext. Evap. ext in K-D unit using 3-ball Snyder column on 55° water bath until vol. is reduced to 3–4 mL.

Let K-D unit cool to room temp., allowing excess solv. in Snyder column to rinse down walls of unit into 4 mL K-D tube (total = 3–4 mL). Remove 250 mL reservoir and 3-ball Snyder column, reduce vol. of ext to 2 mL in same K-D tube under gentle stream of N (ca 50 mL/min), and transfer 2 mL ext using disposable Pasteur pipet with two 1 mL mineral oil rinses to 50 mL purge and trap app. contg 20 mL mineral oil and 1 mL of 10 mg/mL of α-tocopherol in mineral oil as nitrosation inhibitor.

Assemble purge and trap app. and connect cartridges to exit tubes with Tygon connector. Adjust Ar flow rate to 400 mL/min thru cartridge ± 5% (i.e., 380–420 mL Ar/min). Note: Check flow rate intermittently during purging, especially within first 15 min because of initial increase in temp. of sample. Immerse purge tubes (up to sample line) or to ca 25 mL mark in 150 ± 3° oil bath for 1.5 h. Remove cartridge and tightly cap. (Note: This is good stopping point; cartridge can be eluted on following day if necessary.)

Elute cartridge using 10 or 20 mL glass Luer-Lok syringe connected to female Luer adapter (air exit side) with 20 mL acetone–CH_2Cl_2 (1 + 1, v/v). Collect eluate in 30 mL culture tube. (Note: 30 mL tube(s) should be scored with file or piece of tape placed at 5 mL vol. mark.)

Evap. ext to ca 5 mL and then transfer with three 1 mL rinses of CH_2Cl_2 to 10 mL graduated tube. Add 0.5 mL keeper soln *2*. Evap. sample (vol. = 8.5 mL) to 2 mL under gentle stream of N. (Note: If 2 mL sample cannot be analyzed same day as evapd, it is advantageous to refrigerate sample at larger vol., i.e., 4–5 mL, and evap. next day before analysis by GC-TEA.)

Analyze 2 mL sample by injecting 8 μL aliquot into GC-TEA.

5.B06 *Quantitation*

Use internal std technic. Dil. external stock std soln with CH_2Cl_2 to 50, 100, and 200 ng/mL to be used as working stds for analysis. Inject 8 μL into GC-TEA to det. responses (peak hts) of NDPA and other nitrosamines for use in internal stdzn calcn. Inject 8 μL of each 2 mL sample ext into GC-TEA. Det. responses (peak hts) of NDPA and any other *N*-nitrosamines detected for use in internal stdzn calcn. Calc. results as follows:

$$\text{ppb } N\text{-Nitrosamine X} = [(PH_X) \times (F_X) \times (100 \text{ ng NDPA})]/[(PH_{NDPA}) \times (F_{NDPA}) \times (\text{g sample})]$$

where PH_X = peak ht in mm of *N*-nitrosamine X in sample; F_X = ng *N*-nitrosamine X/mL in external std soln divided by peak ht in mm of *N*-nitrosamine X in external std soln; 100 ng NDPA = total ng NDPA (internal std) added to sample; PH_{NDPA} = peak ht in mm of NDPA (internal std) in sample; F_{NDPA} = ng NDPA/mL in external std soln divided by peak ht in mm of NDPA in external std soln; g sample = g rubber sample analyzed.

Results and Discussion

Complete results were received from 5 laboratories (Table 1). Most collaborators were impressed with the sample cleanup efficiency of the purge and trap step before analysis by GC-TEA. One collaborator reported that the method was more time-efficient than the procedure of Havery and Fazio (11) because multiple samples could be run simultaneously.

The data from the 5 laboratories were used to calculate the statistical summary (13) shown in Table 2. Laboratory 3 was an outlier for composite A by the Cochran test. Even when the values obtained by laboratory 3 for composite A are used, as shown in Table 2, a repeatability relative standard deviation (RSD_o) of 16.1% and a reproducibility relative standard deviation (RSD_x) of 25.2% are obtained, which are consistent with historical values reported by Horwitz (14) for analyte concentrations in the 1–100 ng/g range.

Recoveries of the internal standard (NDPA) used in each analysis by each laboratory are reported in Table 3. Each participant spiked each sample in the first step of the method at a 20 ng/g level. Mean recoveries of NDPA from each of the 3 composites (A, B, and C) were very consistent when data from all 5 laboratories were averaged (n = 20), whereas those from individual laboratories (for samples A, B, and C) varied significantly (n = 12).

Procedural reagent blanks were run by each laboratory and were negative for nitrosamines. Latex rubber pacifier samples that were blank for nitrosamines do not exist and were therefore unobtainable for use in the study.

Only 4 volatile *N*-nitrosamines have previously been detected in latex rubber infant pacifiers (NDMA, NDEA, NPIP, and

Table 3. Recoveries[a] of NDPA internal standard in collaborative study

	Composite A sample set[b]				Composite B sample set				Composite C sample set				
Lab.	1	5	8	11	2	4	7	10	3	6	9	12	$\bar{x}$ ± SD by Lab.
1	95.0	85.0	100	95.0	85.0	85.0	90.0	85.0	85.0	88.9	86.1	90.0	89.2 ± 5.1
2	73.1	89.4	87.5	91.7	90.2	97.0	106	94.4	82.9	94.0	84.7	87.5	89.9 ± 8.1
3	55.7	77.3	72.7	65.9	79.6	69.4	55.7	85.3	81.8	67.1	72.7	67.1	70.9 ± 9.4
4	78.3	87.0	78.3	87.0	78.3	82.6	78.3	69.6	69.6	73.9	91.3	66.2	78.4 ± 7.7
5	92.3	96.4	91.6	89.2	90.6	88.2	88.2	94.1	80.0	98.8	100	94.1	92.0 ± 5.4
$\bar{x}$ ± SD by composite		84.4 ± 11.3				84.6 ± 11.1				83.1 ± 10.6			

[a]Units are % NDPA recovered. Spike level was 20 ng NDPA/g rubber (20 ppb).
[b]Blind replicate numbers.

NDBA). NDBA is the principal nitrosamine found in this product. Since the 60 ppb regulation was issued (January 1, 1984) (15), levels of NDBA in latex pacifiers have ranged from 3.14 to 112 ppb and levels of NDMA from 1.17 to 3.55 ppb (10). No NDEA has been found in latex pacifiers since issuance of the regulation. Before that date, NDEA levels ranged from 1.39 to 7.16 ppb (10). Only one brand of pacifier of those tested for regulation compliance contained NPIP with concentrations ranging from 2.11 to 4.03 ppb (10).

This method was collaborated for NDBA only because samples of latex pacifiers containing NDMA and NDEA in sufficient concentration to allow their analysis were unobtainable. The minimum detectable level (MDL) for NDBA with this method is 3.6 ng/g.

NDMA was detected in composites B and C at levels ≤ MDL (1.5 ng/g). No NDMA was detected in composite A. Therefore, results for NDMA could not be used in the statistical evaluation of the collaborative study data. The method has been previously validated and used in the authors' laboratory for analysis of NDMA and NDEA in latex pacifiers with results comparable to those obtained in the collaborative study. This was achieved by using samples obtained before issuance of the regulation and industry compliance.

For about 1.5 years the method has been used successfully by a highly reputable private analytical laboratory for determination of volatile *N*-nitrosamines. The collaborative study results indicate that the method can be successfully used to support the current 60 ppb action level and any future requirements to levels as low as 7 ppb.

Since volatile *N*-nitrosamines are known animal carcinogens, all manipulations with these chemicals should be performed in a well ventilated fume hood or glove box. Protective gloves should be worn to prevent skin contact. Inhalation of vapors should also be avoided.

Recommendation

The Associate Referee recommends that the GC-TEA method for determination of *N*-nitrosodibutylamine in latex infant pacifiers be adopted official first action.

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MYCOTOXINS

Optimum Methanol Concentration and Solvent/Peanut Ratio for Extraction of Aflatoxin from Raw Peanuts by Modified AOAC Method II

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The amount of aflatoxin extracted from raw peanuts by using the water-slurry modification of AOAC Method II was determined for 49 different combinations of methanol concentration and solvent/peanut ratio. Results indicate that the amount of aflatoxins B_1 and B_2 extracted from raw peanuts is a function of both methanol concentration and solvent/peanut ratio, and a cubic equation was developed, using regression techniques, to describe the combined effects. From the functional relationship, the predicted methanol concentration and solvent/peanut ratio that extracts the most aflatoxin B_1 was computed to be 60.0% and 10.8 mL solvent/g peanuts, respectively. This combination extracted 12.1% more aflatoxin than did AOAC Method II.

AOAC Method II specifies a solvent consisting of 55% methanol in water (v/v) and a solvent/peanut ratio of 5/1 (mL/g) for the extraction of aflatoxin from 50 g samples of raw peanuts (1). Since 1983, the Agricultural Marketing Service (AMS) of the U.S. Department of Agriculture has used a water-slurry method to extract aflatoxin from 1100 g samples of raw peanuts (2). This method has been approved by AOAC as a revision to AOAC Method II (3, 4).

Studies related to the development of the water-slurry method indicated that the methanol concentration of the solvent and/or the solvent/peanut ratio have an effect on the amount of aflatoxin extracted from raw peanuts (3). Sixteen treatment combinations of 4 methanol concentrations (55, 60, 65, and 70% methanol in water) and 4 solvent/peanut ratios (3, 4, 5, and 6 mL solvent per g peanuts) were used in a later study, but it appeared that none of the combinations maximized the amount of aflatoxin extracted (5). The objective of the present study was to determine the combination of methanol concentration and solvent/peanut ratio that maximizes the amount of aflatoxin extracted from raw peanuts when the water-slurry method is used.

Experimental

Aflatoxin-contaminated peanuts were comminuted in a mill similar to that used in AMS aflatoxin laboratories (6). Three 1100 g samples of comminuted peanuts were each blended with 1600 mL water and 22 g sodium chloride in a 1 gal. blender jar at high speed for 3 min. The 3 samples of slurry were mixed in a large container, and 49 portions, each weighing 123.7 g, were removed from the container. Each 123.7 g portion of slurry contained 50 g peanuts, 72.7 mL water, and 1 g sodium chloride. Proper quantities of methanol and water were added to each of the 49 slurry portions to achieve 49 different combinations of methanol concentration and solvent/peanut ratio. The 49 combinations consisted of 7 methanol concentrations (40, 45, 50, 55, 60, 65, and 70% methanol in water) and 7 solvent/peanut ratios (5, 7, 9, 10, 11, 13, and 15 mL solvent per g peanuts). After the appropriate amount of methanol and water was added to each 123.7 g slurry portion, 100 mL hexane was added and the mixture was blended in a 1 qt blender jar at high speed for 30 s. The steps to extract aflatoxin from the blend were the same as those described in AOAC Method II with the following 3 exceptions: *(i)* The quantity of methanol–water solution transferred from the centrifuge bottle to the separatory funnel varied according to the solvent/peanut ratio used for the extraction so that 10 g peanuts was represented in the solution for each of the 49 combinations; *(ii)* the methanol–water solution was filtered through coarse paper to remove peanut particles and oil before the solution was placed in the separatory funnel; and *(iii)* the proper amount of either methanol or water was added to the filtered methanol–water solution in the separatory funnel to adjust the methanol concentration to 55% before the solution was washed with chloroform.

The amount of aflatoxin in each extract was measured using thin layer chromatography (TLC). The intensities of the fluorescent spots on the TLC plate were measured densitometrically (7). Each of the 49 extracts was spotted on one of 4 TLC plates according to an incomplete block design (8). Three TLC plates each contained 14 extracts and the fourth TLC plate contained 7 extracts. This spotting procedure was replicated a total of 4 times using a different assignment pattern for each replication. A total of 16 TLC plates were used to quantitate the aflatoxin in the 49 extracts for a sample. The above procedure was repeated 10 times so that ten 123.7 g slurry portions were extracted by each of the 49 combinations of methanol concentration and solvent/peanut ratio.

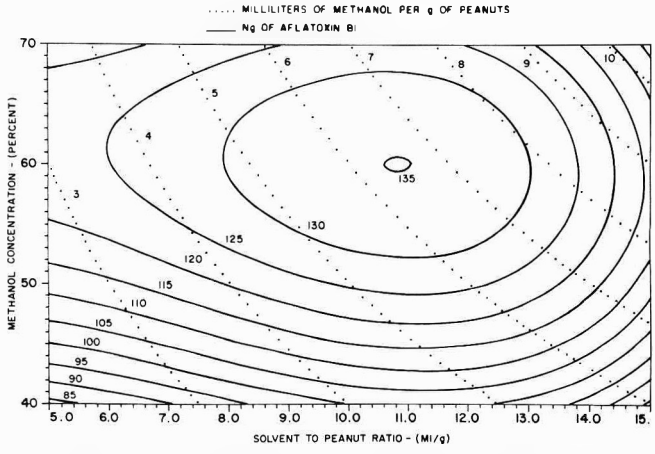

Figure 1. Volume of methanol used for extraction and amount of aflatoxin B_1 extracted per g raw peanuts by water-slurry method.

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Table 1. Amount (ng)[a] of aflatoxins B_1 and B_2 extracted per g peanuts by water-slurry modification of AOAC Method II for each of 49 combinations of methanol concentration and solvent/peanut ratio

Methanol, %	Solvent/peanut ratio, mL/g						
	5	7	9	10	11	13	15
	Aflatoxin B_1						
40	86	91	98	104	109	91	83
45	94	101	108	124	111	108	97
50	119	113	124	128	133	118	108
55	113	119	128	132	142	132	116
60	134	124	126	139	131	125	118
65	124	129	131	139	126	113	112
70	117	114	128	131	130	120	99
	Aflatoxin B_2						
40	19	19	20	21	22	18	17
45	20	21	22	24	21	22	19
50	24	24	26	25	26	24	21
55	23	25	25	25	28	26	23
60	26	26	25	29	27	25	23
65	26	27	26	27	26	22	24
70	25	23	26	26	26	24	20

[a]Each amount is average of 40 determinations (10 samples × 4 TLC determinations per sample). Averages were computed after removal of TLC plate-to-plate variation by using within-plate analysis.

Table 2. Regression coefficients for Equation 3[a]

Aflatoxin (A)	Non-zero coefficients for Equation 3[b]							R^2
	C0	C1	C2	C3	C4	C5	C7	
B_1	−192.005	−8.9325	1.9452	−0.08625	10.6951	−0.0848	−0.04805	0.642
B_2	−33.116	−0.7790	0.2522	−0.01214	1.9086	−0.01494	−0.00980	0.609

[a]$A = C0 + C1 \times S + C2 \times S^2 + C3 \times S^3 + C4 \times M + C5 \times M^2 + C6 \times M^3 + C7 \times M \times S + C8 \times S \times M^2 + C9 \times M \times S^2$.
[b]Coefficients C6, C8, and C9 were not significantly different from 0 at the 5% confidence level.

Results and Discussion

The mL methanol, Q, and the mL water, W, added to each 123.7 g slurry portion (containing 50 g peanuts) is shown in Equations *1* and *2:*

$$Q = (0.5)(M \times S) \quad (1)$$

$$W = 0.55(100 - M) - 72.7 \quad (2)$$

where S is mL solvent per g peanuts and M is the methanol concentration in percent. The average amounts of aflatoxins B_1 and B_2 extracted by each of the 49 treatment combinations are shown in Table 1. The averages in the table were computed after the TLC plate-to-plate variation was removed by using within-plate analysis. Each value in Table 1 represents 40 determinations (10 samples × 4 TLC determinations per sample). The data indicate that the amount of aflatoxin extracted is a function of both methanol concentration and solvent/peanut ratio. Because the peanuts did not contain sufficient amounts of G_1 and G_2, these aflatoxins are not reported.

To mathematically describe the relationship between ng aflatoxin extracted, A, the percent methanol concentration, M, and the solvent/peanut ratio, S, a third degree polynomial was fitted to the B_1 and B_2 data in Table 1, using regression techniques (9):

$$A = C0 + C1 \times S + C2 \times S^2 + C3 \times S^3 + C4 \times M + C5 \times M^2 + C6 \times M^3 + C7 \times M \times S + C8 \times M \times S^2 + C9 \times M^2 \times S \quad (3)$$

where C0 through C9 are coefficients determined by the regression analysis. The coefficients C6, C8, and C9 in Equation *3* were not significantly different from 0 at the 5% confidence level for either B_1 or B_2. The values of the non-zero coefficients in Equation *3* for B_1 and for B_2 are shown in Table 2. The coefficient of determination for the B_1 and B_2 regressions was 0.642 and 0.609, respectively. Equation *3* along with the coefficients shown in Table 2 were used to determine the combination of methanol concentration and solvent/peanut ratio that maximized the amount of aflatoxins B_1 and B_2 extracted. The partial derivatives of Equation *3* with respect to M and with respect to S were each set equal to zero, and the 2 partial derivative equations were solved for M and S. Equation *3* along with coefficients in Table 2 should only be used to predict A for M values between 40 and 70% and for S values between 5 and 15 mL/g. The methanol concentration and solvent/peanut ratio that maximized B_1 and B_2 are 60.0% and 10.8/1, and 60.7% and 10.2/1, respectively. In view of the close agreement between these values and because the concentration of B_1 is usually higher than B_2, it would seem appropriate to use a methanol concentration of 60.0% and a solvent/peanut ratio of 10.8/1.

A plot of B_1 contours for Equation *3* showing constant amounts of extracted aflatoxin B_1 is shown in Figure 1. Also shown in Figure 1 are contours of constant methanol volumes. From the figure, the volume of methanol that was required to extract a given amount of aflatoxin from the raw peanuts used in this study can be determined. In the region near the point where the most aflatoxin was extracted, large changes in either the methanol concentration or the solvent/peanut ratio resulted in small changes in the amount of aflatoxin extracted.

When a methanol concentration of 60.0% and a solvent/peanut ratio of 10.8/1 are used in Equation *3*, the predicted total aflatoxin extracted is 162 ppb (135 ppb B_1 + 27 ppb B_2). When a methanol concentration of 55% and a solvent/peanut ratio of 5/1 are used, as specified by AOAC Method II, the predicted total aflatoxin extracted is 145 ppb (120 ppb B_1 + 25 ppb B_2). The percent difference between the 2 amounts of

aflatoxin extracted from peanuts used in this study is 12.1% ((162 − 145)/145). (The 12.1% was calculated on aflatoxin concentrations computed from Equation *3* to the nearest one tenth of a ppb.) This percent increase requires 2.36 times more methanol than required for AOAC Method II.

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Production and Isolation of Aflatoxin M_1 for Toxicological Studies

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One hundred mg aflatoxin M_1 was produced and purified for toxicological studies. *Aspergillus flavus* NRRL 3251 was cultured on rice to produce aflatoxins B_1, B_2, M_1, and M_2. B_1 and B_2 were separated from M_1 and M_2 by a normal phase low pressure liquid chromatography (LC) column. M_1 was then separated from M_2 by a reverse phase low pressure LC column. Recoveries of aflatoxins from the LC columns were about 90%. The purified M_1 was confirmed by ultraviolet-visible spectrometry, mass spectrometry, nuclear magnetic resonance spectrometry, optical rotation, and its mutagenicity to *Salmonella typhimurium* TA98.

Aflatoxin M_1, a hydroxylated metabolite of aflatoxin B_1, is a widely occurring contaminant of milk and dairy products, but the exact health hazard of M_1 has yet to be assessed. Previous studies have demonstrated the acute toxicity and carcinogenic effects of M_1 (1–3), but its carcinogenicity needs further assessment. In recent years, a great deal of concern has been expressed over the cancer risk posed by long-term, low-level exposure of young human consumers to M_1. Therefore, a method was developed to produce sufficient milligram quantities of M_1 for toxicological studies. A modified version of Stubblefield's method (4) of culturing *Aspergillus flavus* NRRL 3251 on rice to produce aflatoxins was used, because the original method was not readily reproducible in our laboratory. This biological system was preferred to ensure the production of the naturally occurring enantiomer of M_1 and thereby avoid the possibility of reduced potency which has been reported to occur with synthetic, racemic mixtures of M_1 (5). Improved purification techniques using a series of low pressure liquid chromatography (LC) columns eliminated the possible contamination by B_1 as a trace impurity of the final product. This method has been used routinely in our laboratory with reproducible results.

Experimental

Reagents and Apparatus

(a) *Solvents.*—Reagent and nanograde acetone, acetonitrile, chloroform, dichloromethane, anhydrous ethyl ether, hexane, methanol, toluene (J. T. Baker Chemical Co.).

(b) *Silica gel.*—E. Merck 7734, 3% water-deactivated after activation for 2 h in 105°C oven.

(c) *Rice.*—Safeway brand, enriched, long grain.

(d) *Wrist-action shaker.*—Model No. 75 (Burrell Corp., 2223 Fifth Ave, Pittsburgh, PA 15219).

(e) *Chromatographic columns.*—Glass column (100 × 20 mm id) with 200 mL solvent reservoir. Prepacked silica gel 60 Lobar® (310 × 25 mm id) 10608-94 and reverse phase RP-8 Lobar® (310 × 25 mm id) 11804-94 (EM Reagent, EM Laboratories, Inc., 500 Executive Blvd, Elmsford, NY 10523).

(f) *Injector.*—Model 5041 (Rheodyne, Inc., PO Box 996, Cotati, CA 94928).

(g) *Pump.*—Minipump® (Lab Data Control Division, Milton Roy Co., Riviera Beach, FL).

(h) *Pulse dampener.*—Model PD-60-LF (Fluid Metering, Inc., 29 Orchard St, Oyster Bay, NY 11771).

(i) *Detector.*—KRATOS variable wavelength Spectroflow SF 770 (Schoeffel Instrument Corp., InstruSpec, Concord, CA).

(j) *Thin layer plates.*—Silica gel 60 plates, 0.25 mm thickness (EM Laboratories, Inc.).

Production and Extraction

Autoclave Erlenmyer flasks (500 mL, Nalgene) containing 50 g rice, 2% yeast extract, 0.4mM zinc sulfate, and 20 mL deionized water at 15 psi, 121°C, for 20 min. Air-dry flasks 24 h and inoculate with *A. flavus* NRRL 3251 (1×10^6 conidia in 0.1 mL 0.01% sodium lauryl sulfate spore suspension). Shake flasks on wrist-action shaker 8 days at 25–28°C. Extract contents of each flask with 150 mL chloroform and 50 mL deionized water on gyrotory shaker 24 h at room temperature. Repeat, but reduce extraction period to 2 h. Combine chloroform extracts and reduce volume under vacuum; redissolve in dichloromethane. This crude extract contains aflatoxins B_1, B_2, M_1, and M_2.

Cleanup

Prepare cleanup columns as follows: Place small amount of glass wool in bottom of column, add enough anhydrous sodium sulfate to cover glass wool, slurry 10 g silica gel with ca 50 mL dichloromethane and add to column, and cover with 7.5 g anhydrous sodium sulfate. Load crude extract onto column, ca 10 mL per column (containing 10 mg M_1). Drain to top of bed. Add the following solvents in 100 mL aliquots,

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Table 1. Significant peaks of nuclear magnetic resonance spectrum of aflatoxin M_1

Designated proton	Chemical shift, (α) ppm	Band multiplicity
A	6.44	singlet
B	6.79[a]	doublet
C	5.60[b]	doublet
D	6.55	singlet
E	2.50	unresolved multiplet
F	3.35[c]	—
G	3.96	singlet
H	6.77	singlet

[a,b]J = 2.71 Hz.
[c]The expected multiplet was masked by a peak resulting from solvent contamination by water.

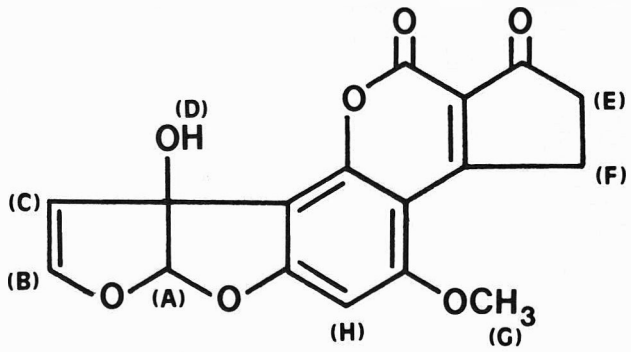

Figure 1. Proton designations for NMR spectrum of aflatoxin M_1.

draining to top of bed after each aliquot: 100 mL toluene–acetic acid (9 + 1), 200 mL ether–hexane (3 + 1), 200–300 mL chloroform–acetone (95 + 5) (elutes B_1 and B_2), and chloroform–acetone (4 + 1) (elutes M_1 and M_2). Monitor elution of M_1 by spotting ca 10–20 μL of each fraction on thin layer chromatographic plate. Develop in chloroform–acetone–isopropanol (85 + 15 + 2.5). Examine plate under longwave UV light for characteristic blue fluorescence of M_1. Pool fractions containing M_1 and reduce volume under vacuum.

Purification

The low pressure liquid chromatography (LC) system consists of a dual piston pump, pulse dampener, injector with 5 mL loop, Lobar column, variable wavelength (UV-VIS) detector, and strip-chart recorder. Condition normal phase (NP) column 1 h with dichloromethane–hexane–methanol (75 + 25 + 5). Dichloromethane and hexane are 100% water-saturated. Load cleaned extract in 5 mL aliquots of running solvent (containing 5 mg M_1) onto LC system via injector. At flow rate of 4 mL/min, B_1 retention time is ca 45 min and M_1 + M_2 retention time is ca 100 min. Use hand-held UV viewing lamp to monitor chromatographic development of aflatoxins. Collect M_1 + M_2 fraction and reduce volume under vacuum. After each use, recondition NP column by inverting column and adding ca 2 bed volumes of methanol, hexane, and dichloromethane, respectively. For each injection onto reverse phase (RP-8) column, dissolve 1–1.5 mg M_1 in 3 mL acetonitrile. Sonicate and add 2 mL water; sonicate again. Condition RP-8 column with water–acetonitrile (75 + 25) for 1 h. RP-8 column separates M_1 from M_2 and removes any trace of contaminating B_1. Retention times for M_2 and M_1 are ca 75 and 95 min, respectively, at flow rate of 3 mL/min. Monitor chromatographic development of M_1 and M_2, using UV-VIS detector at 357 nm. Collect M_1 and extract with equal volume of chloroform 3 times; reduce volume under vacuum. After each use, invert RP-8 column and recondition by flushing with ca 2 bed volumes of methanol–dichloromethane (9 + 1). The M_1 purified by the RP-8 column was used as such in toxicity assays without crystallization.

Confirmation of Aflatoxin M_1

The purity of M_1 was examined by TLC and identity of the purified M_1 was confirmed by UV-VIS spectrometry, mass spectrometry, and NMR spectrometry and by co-chromatography with authentic M_1. The UV-VIS spectrum in methanol (λ_{max}:225, 265, 357 nm) was determined on a Cary 15 spectrophotometer (Varian). Mass spectral analysis was done on a GC/MS/DS Finnigan 3200 E mass spectrometer. Two hundred ng M_1 was concentrated in a glass capillary and inserted as a solid probe. Sensitivity was 10, EM1350, filter 100, temperature 290°C, and scan rate 3 s/scan. The parent ion 328 and the following fragment ions 299, 271, and 243 were evident. The proton NMR spectrum of M_1 was obtained on an NT360 MHz (Nicolet Magnetics) spectrometer at 23°C; 4 mg M_1 was dissolved in deuterated dimethyl sulfoxide (DMSO-D6, spectroscopic grade). The significant peaks and corresponding protons are listed in Table 1, Figure 1. Our data support previously reported data (6). Specific rotation of M_1 was confirmed in dimethylformamide (spectroscopic grade) according to Holzapfel and Steyn (6), at a concentration of 0.5 mg/mL, to give $[\alpha]_D$ −300° at 20°C. The mutagenicity of M_1 was compared to that of B_1 in a microsuspension assay (7), a modification of the Ames *Salmonella/* microsome mutagenicity test (8). The microsuspension assay incorporates a preincubation of test compound, bacteria and S-9 mix, and a 10-fold concentration of bacteria, resulting in much increased sensitivity. Both B_1 and M_1 were tested in DMSO (spectroscopic grade), with and without S-9 (Aroclor 1254-induced), using *S. typhimurium* TA98. The slopes and correlation coefficients as determined by linear regression were 0.57 revertants/ng, $r = 0.97$ for M_1, and 61.5 revertants/ng, $r = 0.99$ for B_1, giving M_1 a mutagenic potency approximately 1/100 that of B_1 (Figure 2).

Safety Precautions

The aflatoxin-producing cultures were incubated in an isolated, constant-temperature room. All extraction and isolation procedures were performed in chemical fume hoods with appropriate ventilation. Carcinogens were handled in solutions throughout the operation. Weighing of solid carcinogens, when needed, was done in a glove-box. All laboratory personnel who handled carcinogens wore mandatory protective clothing and gloves. Any possible spillage of aflatoxin solutions was frequently monitored by a hand-held UV viewing lamp. Fluorescent spots were immediately treated with 10% NaClO solution.

Results and Discussion

The system we have developed represents a relatively safe, efficient one for producing and purifying milligram quantities of M_1. The average yield of the 3 best runs was 20 mg pure M_1 per kg rice. The identity of this M_1 produced by the *A. flavus* cultures was rigorously confirmed using UV-VIS, MS, and NMR spectroscopy. This fungal product was indistinguishable from M_1 produced through biotransformation of B_1 with rat hepatic microsomes (9), when compared by 2-dimensional TLC and specific optical rotation (levorotatory form). The mutagenicity of the M_1 produced in this study to that of B_1 is 1/100 and is lower than values previously reported for rat S-9 of 1/30 (10) and trout S-9 of 1/63 (11). The reduced potency of our product is probably attributable to its increased

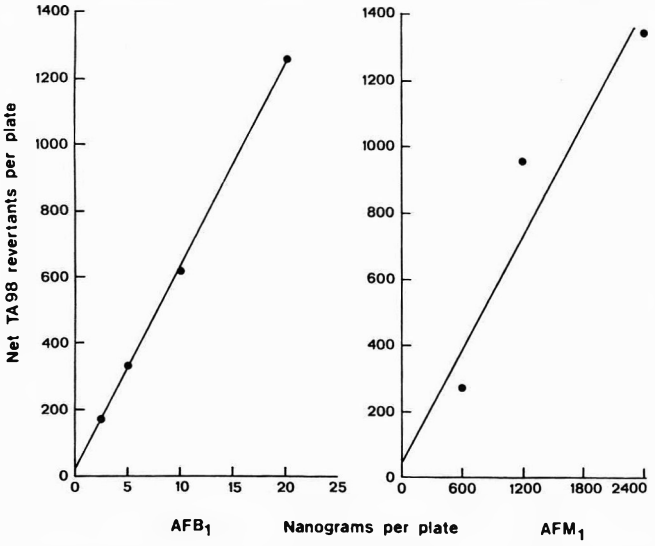

Figure 2. Comparative mutagenicity of aflatoxins B_1 and M_1, in DMSO, with S-9. Each point is average of 3 plates, minus background revertants. For B_1, slope = 61.5 revertants/ng, r = 0.99; for M_1, slope = 0.57 revertants/ng, r = 0.97.

purity due to the use of reverse phase chromatography which eliminates the possibility of B_1 contamination since M_1 is eluted first. Part of the quantities of M_1 so purified was used in a chronic feeding experiment for the assessment of the carcinogenicity of M_1 in the male Fischer rat. The hepatocarcinogenicity of this product to the rat was determined to be 2–10% that of B_1 (12).

The capacity of the column system described in this communication is limited by 2 factors: the amounts of interfering impurities in the crude extract of M_1 and the solubility of M_1 in the water–acetonitrile (75 + 25) solution. M_1 is a minor secondary metabolite of the *A. flavus* culture. The crude extract of M_1 contained B_1 in excess of 30 times the amount of M_1; other fungal product impurities conceivably were present in even greater quantities. The capacity of the RP-8 column was limited by the solubility of M_1 in the reverse phase eluant. Despite these limitations, this column system has offered an effective means to obtain sufficient quantities of pure M_1 for toxicological studies.

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DRUGS

Colorimetric Determination of Certain Phenothiazine Drugs by Using Morpholine and Iodine-Potassium Iodide Reagents

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A colorimetric method was developed for the quantitative estimation of 11 phenothiazine drugs. The method is based on the interaction of unsulfoxidized drug with morpholine and iodine–potassium iodide reagents. The interaction for all studied phenothiazine drugs yields a blue product with 2 absorption maxima: one in the range of 620–640 nm with lower molar absorptivity and the other in the range of 662–690 nm with higher molar absorptivity. The color was stable for at least 10 h. The reproducibility and recovery of the method were excellent. The method was applied successfully to the analysis of various commercially available phenothiazines in different dosage forms. The results were comparable to those obtained by official procedures. The suitability of the method for detection and estimation of promethazine excreted in urine has been suggested by preliminary experiments. Reaction products have been isolated and identified.

Numerous methods for, and excellent reviews on, the analysis of phenothiazines are available in the literature (1–3). Among the methods used to assay phenothiazine drugs in bulk as well as in pharmaceutical preparations and biological fluids are titrimetric (4, 5), chromatographic (6–9), electrochemical (10, 11), ultraviolet (4, 12, 13), and visible spectrophotometric (14–17). Because many of these procedures suffer interference from excipients, coloring and flavoring agents, or oxidation products of phenothiazine drugs, we decided to develop a new method to overcome these interferences.

A spot test method was described for detection of secondary amines by formation of methylene blue-like dyestuffs, using phenothiazine and bromine as reagents (18). The applicability of the principle of this reaction for quantitative determination of phenothiazines has been investigated using morpholine and iodine–potassium iodide as reagents. As a result of this investigation, a rapid, sensitive, and selective colorimetric method for determination of 11 phenothiazine drugs has been developed.

METHOD

Apparatus and Reagents

(a) *Ultraviolet-visible spectrophotometer.*—PM2 DL (Zeiss, Oberkochen, GFR).

(b) *Chemicals.*—Pharmaceutical grade: phenothiazine base, promethazine HCl, promazine HCl, alimemazine tartrate, mepazine HCl, perazine maleate, prochlorpromazine maleate, chlorpromazine HCl, levomepromazine maleate, thiethylperazine maleate, thioridazine HCl, and oxomemazine tartrate were obtained as gifts from various manufacturers and were used as working standards without further treatment. Promethazine sulfoxide was prepared by a reported procedure (19). All solvents used throughout this work were analytical grade.

(c) *Iodine-potassium iodide solution.*—Into 100 mL volumetric flask, add 1 g iodine to 10 mL water containing 1 g potassium iodide. After complete dissolution, dilute solution to mark with isopropanol. Mix well, and store 24 h before use.

(d) *Morpholine solution.*—3% v/v morpholine in water.

(e) *Dosage forms.*—Various commercial preparations purchased from local sources.

Preparation of Standards

Dissolve accurately weighed amount of each phenothiazine drug as free base or its salt in methanol and dilute quantitatively with same solvent to obtain appropriate dilutions for each drug (Table 1).

Preparation of Samples

Tablets.—Weigh 20 tablets and finely powder. Transfer accurately weighed amount of powder equivalent to 25 mg of each drug to 100 mL measuring flask and dilute to mark with methanol. Shake mixture well and filter. Discard first portion of filtrate. Use clear solution obtained as stock solution. Dilute stock solution quantitatively with methanol to obtain 10, 50, and 20 μg/mL of promethazine HCl, chlorpromazine HCl, and thioridazine HCl, respectively. Use these solutions as final sample dilutions.

Liquid preparations (syrups, vials, and drops).—Dilute accurately measured volume of each preparation equivalent to 25 mg declared drug quantitatively to obtain 10, 20, and 50 μg/mL of promethazine HCl, promazine HCl, and chlorpromazine HCl, respectively. Use these solutions as final sample dilutions.

Injection.—Mix well the contents of 10 ampoules. Dilute accurately measured volume of solution equivalent to 50 mg chlorpromazine HCl quantitatively with methanol to obtain 50 μg chlorpromazine HCl/mL. This is final sample dilution.

Recovery study.—Add accurately weighed amount of declared drug for each preparation to 100 mL volumetric flask, containing accurately weighed quantity of the powdered tablets or accurately measured volume of liquid preparations or injections. Either dissolve contents of flask in methanol and treat as described for tablets, or dilute quantitatively with methanol to obtain required concentration as described for liquid preparations and injections.

Determination

To 1 mL of either standard or sample phenothiazine solution in 10 mL volumetric flask, add 1 mL morpholine solution followed by 1 mL iodine-potassium iodide solution. Heat mixture on boiling water bath 5 min. Let cool, dilute solution to volume with isopropanol, and let stand 10 min. Measure absorbance of solutions at specified $\lambda(max_2)$ for each phenothiazine drug (Table 1) against blank prepared as described above, except take 1 mL methanol instead of standard or sample solution.

Determination of Promethazine HCl in Urine

To 1 mL of either promethazine HCl or urine in separate 10 mL volumetric flasks, add 1 mL morpholine solution followed by 1 mL iodine-potassium iodide solution. Proceed as

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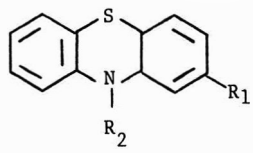
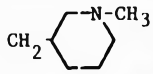
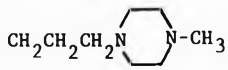
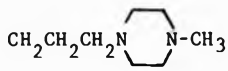
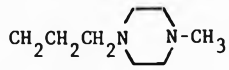
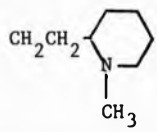
Table 1. Absorption characteristics for the reaction products



Drug	R_1	R_2	Concn,[a] μg/mL	λ_{max_1}	ε_1	λ_{max_2}	ε_2
Phenothiazine base	H	H	8	620	3.0×10^4	662	5.3×10^4
Promethazine HCl	H	$CH_2CH(CH_3)N(CH_3)_2$	10	620	4.2×10^4	662	7.2×10^4
Promazine HCl	H	$CH_2CH_2CH_2N(CH_3)_2$	20	620	1.6×10^4	662	2.7×10^4
Alimemazine tartrate	H	$CH_2CH(CH_3)CH_2N(CH_3)_2$	80	620	7.1×10^3	662	1.1×10^4
Mepazine HCl	H		20	620	1.5×10^4	662	2.4×10^4
Perazine maleate	H		200	620	2.2×10^3	662	3.7×10^3
Prochlorperazine maleate	Cl		400	620	1.6×10^3	665	2.3×10^3
Chlorpromazine HCl	Cl	$CH_2CH_2CH_2N(CH_3)_2$	50	620	1.0×10^4	665	1.2×10^4
Levomepromazine maleate	OCH_3	$CH_2CH(CH_3)CH_2N(CH_3)_2$	100	630	6.2×10^3	680	7.8×10^3
Thiethylperazine maleate	SC_2H_5		300	630	2.1×10^3	680	2.8×10^3
Thioridazine HCl	SCH_3		20	640	1.4×10^4	690	1.9×10^4

[a] In the final solution.

under *Determination*. Transfer resulting turbid blue solution to centrifuge tube and centrifuge 5 min. Measure clear blue solutions at λ(max) 662 nm against blank treated concurrently.

Experimental

Isolation and Characterization of Reaction Product

Insoluble picrate of phenothiazine.—About 300 mL colored reaction product of phenothiazine base was prepared. To this solution, excess saturated aqueous solution of picric acid was added portionwise with vigorous stirring and the mixture was kept in a refrigerator for 24 h. The precipitated picrate salt was filtered and washed with water several times, dried, and recrystallized from aqueous ethanol to give amorphous dark blue powder with mp 171–174°C, λ(max) 662 nm. IR spectrum: no N-H absorption in the 3500–3300 cm^{-1} region. Analysis: Calculated for $(C_{26}H_{24}N_6O_9S)_2 \cdot H_2O$: C, 51.6; H, 4.14; S, 5.3. Found: C, 51.4; H, 4.6; S, 5.8.

Insoluble perchlorate of phenothiazine.—The above procedure was carried out using 0.1M perchloric acid instead of saturated solution of picric acid. After recrystallization from aqueous ethanol, a dark blue amorphous powder was obtained, mp 210–214°C, λ(max) 660 nm. IR spectrum: no N—H absorption in the 3500–3300 cm^{-1} region. Analysis: Calculated for $C_{20}H_{22}N_3O_6SCl.2H_2O$: C, 47.66; H, 5.17; S, 6.4; Cl, 7.5. Found: C, 47.70; H, 5.00; S, 6.8; Cl, 7.1.

Recovered base of insoluble picrate or perchlorate phenothiazine.—About 0.2 g picrate or perchlorate salts of phenothiazine colored reaction product was suspended in 50 mL 2M aqueous sodium hydroxide in a 250 mL separatory funnel. The suspension was extracted 5 times each with 10 mL chloroform. The combined extracts were evaporated to dryness under reduced pressure. A dark blue amorphous precipitate was obtained, mp 175–177°C, λ(max) 662 nm.

Thin Layer Chromatography Study

TLC precoated (0.1 mm) aluminum cellulose sheets, 20 × 20 cm (without fluorescent indicator, E. Merck, Darmstadt, GFR), were used.

Ten μL each of methanolic solutions of standard phenothiazine base, phenothiazine base reaction mixture, and

Table 2. Comparative summary of some statistical data

Drug	Linear cal. range at $\lambda(max_2)$, μg/mL	Slope	Intercept	Correlation coefficient
Phenothiazine base	0.1–4	0.2689	0.0057	0.9995
Promethazine HCl	0.2–4	0.2076	0.0243	0.9958
Promazine HCl	0.5–12	0.0245	0.0063	0.9992
Alimemazine tartrate	2.0–40	0.0245	0.0063	0.9992
Mepazine HCl	0.5–16	0.0652	0.0043	0.9999
Perazine maleate	5.0–100	0.0081	0.0061	0.9997
Prochlorperazine maleate	10.0–200	0.0045	0.0071	0.9985
Chlorpromazine HCl	1.6–30	0.0344	0.0080	0.9998
Levomepromazine maleate	3.0–60	0.0174	0.0018	0.9999
Thiethylperazine maleate	8.0–200	0.0053	0.0067	0.9995
Thioridazine HCl	1.0–25	0.0465	0.0010	0.9999

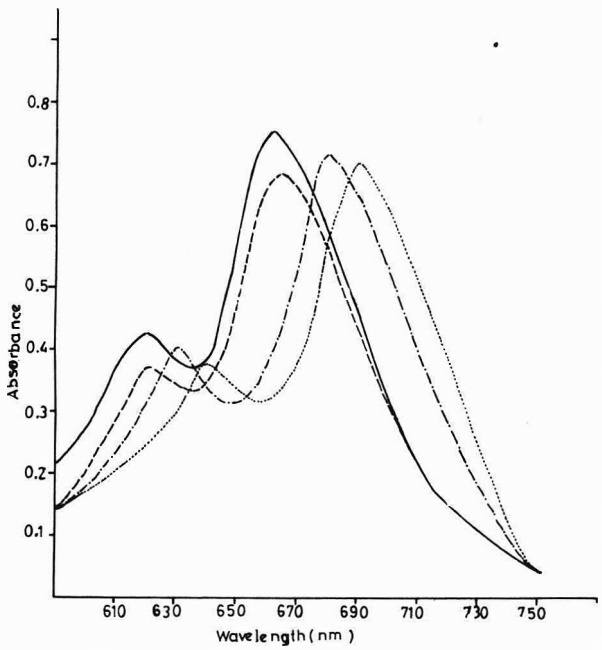


Figure 1. Absorption spectra of colored product of promethazine HCl (_____), chlorpromazine HCl (- - -), levomepromazine maleate (-·-·-), and thioridazine HCl (· · · ·).

methanolic solution of recovered base of insoluble picrate and perchlorate phenothiazine reaction product were spotted on the same TLC plate. The plate was developed in a saturated tank containing ammonium acetate–water–methanol (3 + 20 + 100, w/v/v) (20). Another TLC plate spotted in the same manner was developed in a saturated tank containing ammonia–methanol (1.5 + 100, v/v) (20). After development to 12 cm, plates were air-dried and colors were examined in daylight and under a UV lamp.

Results and Discussion

The absorption spectra for the blue products of the phenothiazine drugs reacted with morpholine and iodine-potassium iodide reagents exhibit 2 λ(max(s)) with different intensity of absorption (Table 1 and Figure 1). Most phenothiazine drugs gave absorption maxima at 620 and 662 nm. A red shift for both λ(max(s)) was observed with levomepromazine maleate and thiethylperazine maleate (λ(max) 630 and 680 nm), while λ(max(s)) for thioridazine HCl shifted to 640 and 690 nm. The shorter wavelength peaks show lower absorption intensity compared to that of longer ones. Thus, measurement was conducted at the longer wavelength throughout this work. Beer's law was obeyed for all phenothiazine drugs studied at their corresponding λ(max). Table 2 shows typical linear regression correlation for all drugs studied.

Effect of Morpholine Concentration

Figure 2 shows the effect of concentration of morpholine solution on the color intensity of the reaction product at $\lambda(max_2)$ for promethazine HCl, levomepromazine maleate, and thioridazine HCl. It is evident that highest color intensity is obtained by using morpholine solution in concentrations ranging from 1.5 to 4%. Therefore, 3% morpholine solution was used in all subsequent work.

Optimization of Iodine Solution

Numerous trials were performed to select the most appropriate iodine solution for maximum color formation. These included using iodine as saturated iodine solution in water, as 1% w/v iodine solution in methanol, ethanol, or isopropanol, as 1% w/v iodine in aqueous potassium iodide solution. The latter solution was prepared by dissolving 1 g iodine in 10 mL 10% aqueous potassium iodide solution and diluting to 100 mL with either methanol, ethanol, or isopropanol.

It was observed that color formation increased when iodine was combined with potassium iodide solution rather than used alone. This may be attributed to the enhanced reactivity of iodine in the presence of potassium iodide. Color intensity was maximum for iodine dissolved in 10 mL 10% aqueous potassium iodide solution and diluted to 100 mL with isopropanol.

Figure 3 illustrates the effect of iodine concentration on the absorption intensity of the colored products of promazine HCl, levomepromazine maleate, and thioridazine HCl measured at the corresponding $\lambda(max_2)$ for each drug. It is quite clear from this figure that color formation could be maximized by using 0.6–1.2 g% iodine solution. The iodine-potassium iodide solution selected and used throughout this work was 1 g iodine dissolved in 10 mL 10% aqueous potassium iodide solution and diluted to 100 mL with isopropanol.

Table 3. Effect of dilution by different solvents on absorbance intensity of developed color[a]

Solvent	Promazine HCl (662 nm)	Levomepromazine maleate (680 nm)	Thioridazine HCl (690 nm)
Methanol	0.476	0.325	0.427
Ethanol	0.472	0.327	0.430
Isopropanol	0.495	0.345	0.456
n-Butanol	0.460	0.317	0.418
Dioxane	0.453	0.317	0.418

[a]Average of 4 determinations.
Final concentration is 6 μg promazine HCl/mL, 20 μg levomepromazine maleate/mL, and 10 μg thioridazine HCl/mL.

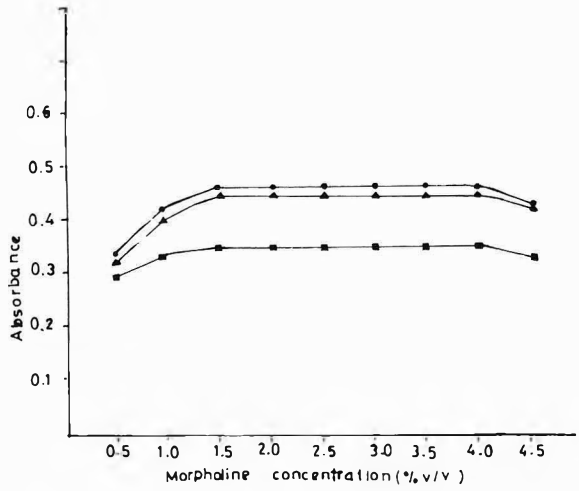


Figure 2. Effect of morpholine concentration on absorption intensity of colored product of 2 μg promethazine HCl/mL (▲), 20 μg levomepromazine maleate/mL (■), and 10 μg thioridazine HCl/mL (●).

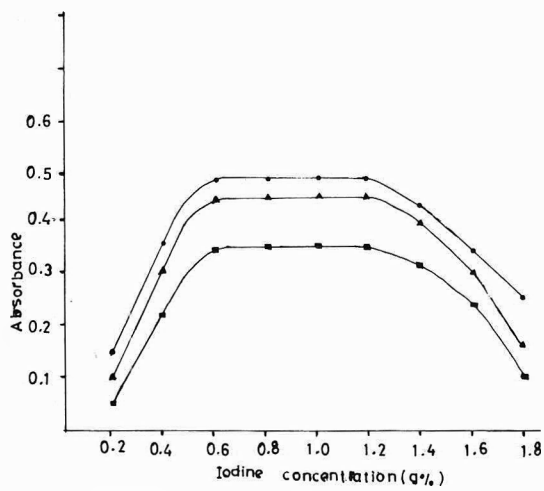


Figure 3. Effect of iodine concentration on absorption intensity of colored product of 6 μg promazine HCl/mL (●), 20 μg levomepromazine maleate/mL (■), and 10 μg thioridazine HCl/mL (▲).

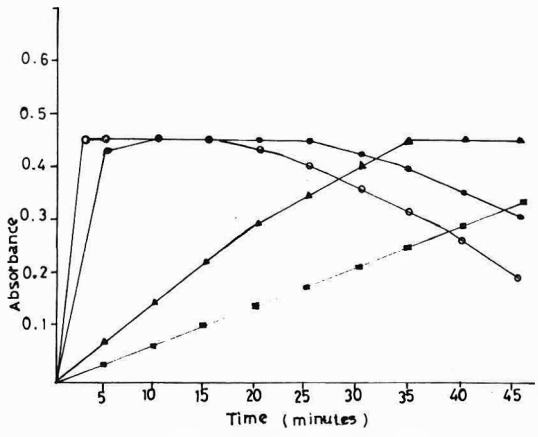


Figure 4. Effect of temperature and reaction time on absorption intensity of colored product of promethazine HCl. Key: (■) ambient temperature 30°, (▲) 60°, (●) 80°, and (○) 100°.

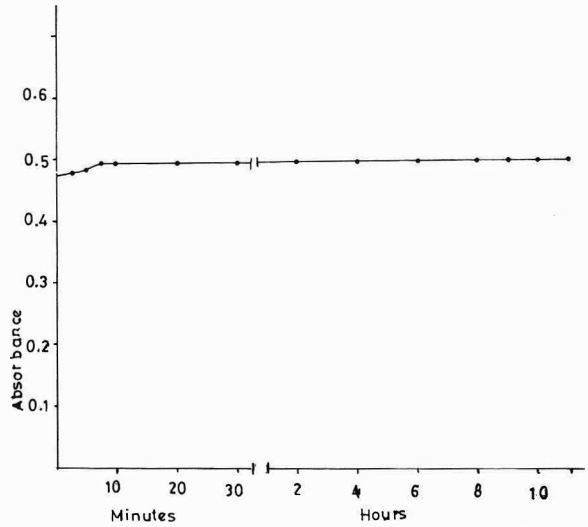


Figure 5. Effect of time on stability of colored product of promethazine HCl (6 μg/mL).

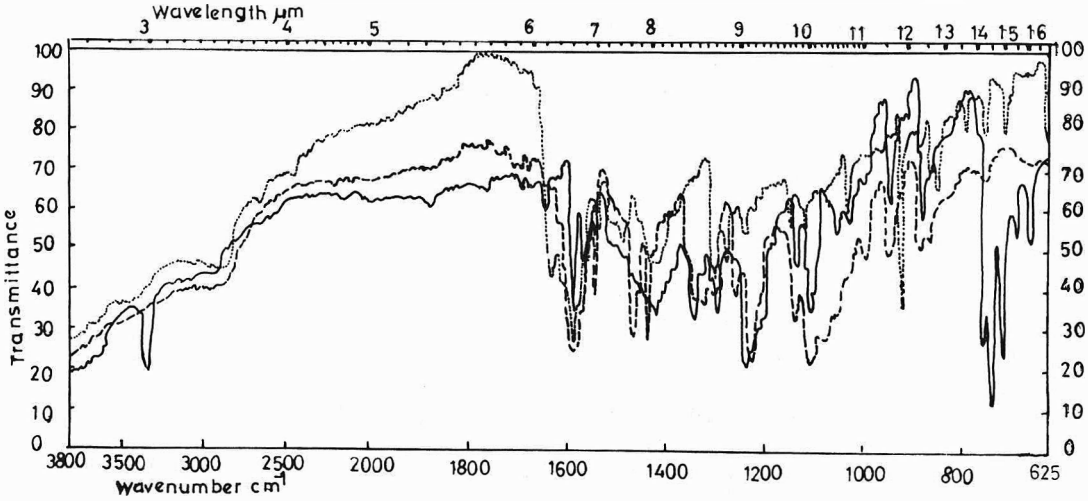
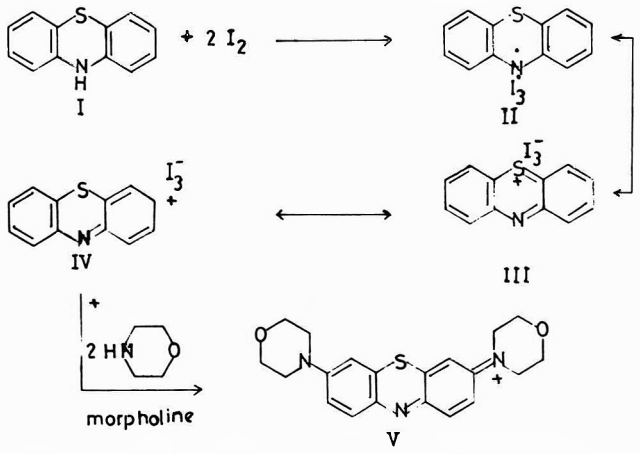


Figure 6. Infrared spectra (KBr disk) of phenothiazine base (_____), picrate salt (· · · ·), and perchlorate salt (- - -) of phenothiazine reaction products.



Scheme 1. Suggested sequence of reaction of phenothiazine (I) with morpholine and I-KI reagent to form phenothiazine dye (V). See text, *Investigation of Reaction Mechanism*, for further discussion.

Effect of Dilution by Different Solvents

Dilution of the colored products by different solvents showed no effect on the position of either λ(max(s)), but the intensity of absorption was influenced slightly. Table 3 indicates that isopropanol is the most suitable diluting solvent because it gave the highest absorption intensity.

Effect of Temperature and Reaction Time

The reaction time was determined by following the color development at ambient temperature (30°C) and in a thermostatic water bath at 60, 80, and 100°C. Figure 4 illustrates the results of this study with promethazine HCl. At 80 or 100°C, absorption was maximum after 10 and 2.5 min, respectively, and remained stable for about 15 min. Further heating decreased the absorption intensity. At 60°C, absorption was maximum after 35 min, while at ambient temperature, the color increased gradually and did not reach maximum intensity until 45 min. Heating in a boiling water bath for 5 min was used throughout this work.

Stability of Color

After dilution of the colored product by isopropanol, a slight increase in absorption was observed in the first 7 min and then remained stable for at least 10 h (Figure 5). Absorbance was measured 10 min after dilution by isopropanol in this study.

Specificity of Reaction

To assess the accuracy of the method in the presence of oxidation products, the concentration of alimemazine tartrate was determined in several standard solutions containing alimemazine tartrate and oxomemazine tartrate; also the concentration of promethazine HCl was determined in several standard solutions containing promethazine HCl and promethazine sulfoxide. Excellent recovery (99.6–100.2%) of the intact alimemazine tartrate and promethazine HCl in these mixtures confirms that the assay is specific for unchanged drug in the presence of its degradation products.

The proposed method was applied for the determination of promethazine HCl, promazine HCl, chlorpromazine HCl, and thioridazine HCl as the drug entity in various pharmaceutical formulations. Recovery experiments were carried out for each drug in its respective pharmaceutical formulations. The results were compared with those obtained by applying the BP method (21). As shown in Table 4, the results are in good agreement and the recovery experiment indicates the absence of interference from frequently encountered excipients, additives, or coloring matters.

Analysis of Promethazine HCl in Urine

A preliminary investigation was carried out for detection and estimation of promethazine HCl in urine. Extraction studies were performed on urine of normal male persons who received 100 mg promethazine HCl in a single dose. The urinary concentration of promethazine HCl was determined in the first 24-h urine collections by the proposed method. The results revealed that only 3.25–3.62 mg of the administered dose was excreted as intact drug in the first 24 h. Intact promethazine HCl was detectable in urine of the subsequent 5 days as evaluated by giving a faint blue color when the urine was subjected to the reaction procedure.

To evaluate the interference of urine components in the assay procedure, as well as the metabolic products of promethazine HCl, freshly made aqueous standard promethazine HCl was added to the first 24-h urine collection. The mixture was allowed to incubate at room temperature for 20

Table 4. Analysis of some phenothiazine drugs in commercial preparations by proposed method and BP method[a]

				Proposed method					
				Found			Recovery		BP method, found, % ± SD
Product	Source	Content	Claimed, mg	mg	% ± SD	Added, mg	mg	% ± SD	
Phenergan tab.	Specia, France	Promethazine HCl	25/tab.	24.54	98.16 ±0.78	25	24.87	99.48 ±0.25	97.15 ±0.54
Promantine syrup	Misr, Egypt	Promethazine HCl	6/5 mL	5.77	96.16 ±0.88	6	5.88	98.00 ±0.54	—[b]
Sparine vial	Wyeth, USA	Promazine HCl	50/mL	48.75	97.50 ±0.33	50	49.00	98.00 ±0.26	97.70 ±0.98
Promacid tab.	CID, Egypt	Chlorpromazine HCl	25/tab.	26.15	104.60 ±0.63	25	25.68	102.72 ±0.23	104.50 ±1.22
Neurazine drops	Misr, Egypt	Chlorpromazine HCl	40/mL	40.80	102.00 ±0.77	40	40.48	101.12 ±0.48	—[b]
Neurazine injection	Misr, Egypt	Chlorpromazine HCl	50/amp.	49.30	98.60 ±0.25	50	49.57	99.14 ±0.96	97.35 ±0.83
Mellerll tab.	Sandoz, Switzerland	Thioridazine HCl	25/tab.	25.93	103.72 ±0.23	25	25.39	101.54 ±0.72	104.45 ±1.06

[a]Average of 5 determinations.
[b]Not official.

Table 5. Spot colors, R_f values and λ(max) of eluted spots of products of phenothiazine base reacted with morpholine and iodine–potassium iodide reagents

Color	R_f[a]	R_f[b]	λ(max), nm
Blue	1.00	1.00	662
Blue	0.90[c]	0.75	662
Blue	0.85	0.70[c]	662
Blue	—	0.45	662
Bluish violet	0.65	0.94	620
Violet	0.50	—	565

[a]Ammonium acetate–water–methanol (3 + 20 + 100, w/v/v).
[b]Ammonia–methanol (1.5 + 100, v/v).
[c]Major blue spot.

min before analysis. One mL urine was carried through the proposed method. Recovery (average of 5 experiments) was greater than 91%.

These findings indicate the absence of interference from biological substances present in urine as well as the specificity of the method for determination of intact promethazine in the presence of its sulphoxide (major metabolic product).

Investigation of Reaction Mechanism

A suggestion for the sequence of reaction is shown in Scheme 1. Phenothiazine (I) interacts first with iodine to form the charge transfer complex (II) followed by total transfer of 2 electrons (III) and the phenothiazine is oxidized to the corresponding phenazathionium periodate (IV). The lowest electron density in the phenazathionium cation at positions C-3, C-7, and S-5 permits the nucleophilic attack at positions 3 and 7 by the unoxygenated agents such as amines (22) (morpholine in this reaction) to give phenothiazine dye (V), analogous to methylene blue.

To confirm this suggestion, the interaction product of phenothiazine base with morpholine and iodine–potassium iodide reagent was subjected to TLC which gave 5 spots with different R_f values and various colors. Methanolic elution of each blue spot gave the same λ(max), while the other spots gave different λ(max) values (Table 5). This indicates that the color complex is not a simple one. Table 5 shows that the chromogen measured at 662 nm is a mixture of at least 4 components.

The identity of the isolated recovered base of phenothiazine reaction product formed under the assay conditions was established by TLC. It gave a single blue spot; its R_f value and λ(max) match that of the major blue spot (Table 5).

The elementary analysis of the perchlorate and picrate salts of phenothiazine reaction products are in agreement with the suggested structure (V). Further evidence supporting our suggestion can be detected from the IR spectra (Figure 6). They reveal 2 characteristic features: first, the disappearance of NH band at 3500–3300 cm^{-1}, and second, the appearance of characteristic CH_2 stretching bands at 2850 cm^{-1} and bending vibration bands at 1430 and 1280–1250 cm^{-1} (23).

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Liquid Chromatographic Determination of Chlorpropamide in Tablet Dosage Forms: Collaborative Study

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A reverse-phase liquid chromatographic method was developed for determining chlorpropamide in tablet dosage forms. Linearity was established over the range 0.2–2.0 μg at a wavelength of 240 nm. The Associate Referee obtained a mean recovery for a synthetic tablet mixture of 99.2%, with a relative standard deviation (RSD) of 1.41. For an authentic tablet mixture, collaborators obtained a mean recovery of 99.6% with an RSD of 0.60%. RSDs were 1.24% for 250 mg/tablet commercial product and also for 100 mg/tablet commercial product. The method has been adopted official first action.

Chlorpropamide, 1-[(*p*-chlorophenyl)sulfonyl]-3-propylurea, is an oral hypoglycemic agent. Its synthesis usually starts with *p*-chlorobenzenesulfonamide (PCBS)(1). Chlorpropamide hydrolyzes to form PCBS, propylamine, and di-*n*-propylurea (2). The relative amounts of the latter 2 compounds are pH-dependent.

The USP XXI assay method for chlorpropamide tablets (3) involves separation by extraction and determination by UV spectrophotometry. This assay is nonspecific and nonstability-indicating. The precursor and degradation product PCBS has an absorption maximum at 228 nm, while the maximum for chlorpropamide is at 232 nm. The absorptivity of PCBS is 25% greater than that for chlorpropamide at 232 nm.

A liquid chromatographic (LC) method (4) was investigated as a specific and stability-indicating assay for chlorpropamide. This method was modified to form the basis of the method subjected to collaborative study. It involves a C_{18} bonded reverse-phase column, a mobile phase of 1% acetic acid and acetonitrile, and a UV detector operated at 240 nm.

The study reported here was initiated as part of the Compendial Monograph Evaluation and Development program of the Food and Drug Administration. The program was designed to evaluate, develop, or improve analytical methods to ensure that they are suitable for regulatory use.

Collaborative Study

To evaluate the proposed LC method, 6 collaborators were sent ground composites of the following 3 samples, each in blind duplicate:

Sample 1: An authentic mixture formulated to contain 532.2 mg chlorpropamide/g.

Sample 2: One lot of commercial tablets labeled to contain 250 mg chlorpropamide/tablet (average tablet weight, 0.4531 g).

Sample 3: One lot of commercial tablets labeled to contain 100 mg chlorpropamide/tablet (average tablet weight 0.1849 g).

In a preliminary study, the Associate Referee tested 5 columns, and obtained the performance parameters listed in Table 1. The Associate Referee also obtained recoveries by the proposed method on 4 commercial samples and one synthetic mixture, with the results shown in Table 2. Four analysts other than the collaborators (2 in the same laboratory as the Associate Referee and 2 in other laboratories) made a preliminary study of the method, using 240 nm as the detection wavelength. Analysts 1 and 2 used Bondapak C_{18} columns and Analysts 3 and 4 used Zorbax ODS columns. Results are also given in Table 2. Agreement from lot to lot was excellent.

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

Chlorpropamide in Drug Tablets
Liquid Chromatographic Method
First Action

37.B01 ***Principle***

Chlorpropamide is dissolved in mobile phase and detd by liq. chromatgy with UV detection at 240 nm.

37.B02 ***Apparatus***

(a) *Liquid chromatograph.*—Equipped with sampling valve capable of introducing 20 μL injections, UV detector capable of operating at 240 nm, and recorder/integrator.

(b) *Column.*—Zorbax ODS, 5–6 μm diam. spherical particles, 4.6 mm × 25 cm (E.I. Dupont, or equiv.).

(c) *Filters.*—Millipore type HVLP, 0.45 μm porosity (Millipore Corp.), or equiv.

37.B03 ***Reagents***

(a) *Mobile phase.*—52/48 ratio of aq./org. phases: (*1*) *Aqueous.*—Acetic acid–H_2O (1 + 99). (*2*) *Organic.*—LC grade CH_3CN.

(b) *Chlorpropamide std soln.*—Transfer ca 50 mg, accurately weighed, USP Chlorpropamide RS to 100 mL vol. flask and dissolve in mobile phase. Dil. quant. to final concn of ca 0.05 mg/mL in mobile phase.

(c) *Resolution soln.*—Chlorpropamide + *p*-chlorobenzenesulfonamide (PCBS) (ca 0.05 mg/mL of each) in mobile phase.

37.B04 ***Preparation of Sample***

Transfer accurately weighed portion of finely ground tablets equiv. to 45–55 mg chlorpropamide to 100 mL vol. flask. Add ca 70–80 mL mobile phase and shake thoroly 6–8 min (or sonicate 3–4 min) and dil. to vol. with mobile phase. Dil. quant. to final concn ca 0.05 mg/mL in mobile phase. Filter portion thru 0.45 μm filter for LC analysis.

37.B05 ***System Suitability***

Set mobile phase at flow rate ca 1.5 mL/min. Retention time for chlorpropamide should not be <4.0 min. Adjust flow rate and/or solv. ratio (do not exceed 50% CH_3CN) for desired retention time. Column should conform to following performance parameters: theoretical plates (n) not <1500; tailing factor (T) not > 1.5; resolution (R) between chlorpropamide and PCBS not <2.0. Relative std deviation for 4 consecutive std injections should be <2.0%.

37.B06 ***Determination***

Make 20 μL injections of std and samples. Det. peak responses (area or ht) obtained and calc. amt of chlorpropamide:

$$\text{Chlorpropamide, mg/tab.} = (r/r') \times (C/W) \times \text{DF} \times \text{ATW}$$

Table 1. Performance parameters of liquid chromatography columns tested by Associate Referee[a]

Column	Flow rate, mL/min	n	k'	T	R
Bondapak C_{18} (Waters Associates)	1.2	1600	1.1	1.4	2.5
Zorbax ODS (E.I. Dupont)	1.4	2200	1.8	1.2	4.8
Hi-Chrom Reversible (Regis Chem. Co.)	1.7	3400	1.4	1.4	4.6
Micropak MCH-10 (Varian Associates)	1.4	1600	1.1	1.5	2.3
Spherisorb ODS (packed in-house)	1.1	1700	1.1	1.4	2.0

[a]n = theoretical plates; k' = capacity factor; T = tailing factor; R = resolution between chlorpropamide and PCBS.

Table 2. Preliminary recoveries of chlorpropamide from commercial tablets (% of label declaration) and a synthetic mixture (% of theoretical) by proposed method

	250 mg/tab.		100 mg/tab.		Synth. mixt. (93.5 mg/183 mg)
	Lot 23	Lot 24	Lot 81	Lot 90	
			Associate Referee		
Av.[a]	99.5	100.5	101.4	101.4	99.2
SD	1.26	1.47	1.82	0.90	1.40
RSD, %	1.27	1.47	1.81	0.89	1.41
			Other Analysts		
Av.[b]	98.8	100.7		101.5	
SD	1.23	1.00		1.48	
RSD, %	1.25	1.00		1.46	

[a]Average of 10 runs.
[b]Average obtained by 4 analysts, each making duplicate runs.

Table 3. Chromatographic parameters obtained by collaborators

Coll.	Column	Solvent ratio, aq./org.	Flow rate, mL/min	Retention time, min	RSD of std injections, %
A	Bondapak C_{18}	54/46	1.5	5.32	0.10
B	Bondapak C_{18}	52/48	1.5	4.25	0.16
C	ASI C_{18} 10	50/50	1.5	4.41	0.16
D	Alltech C_{18} 10	52/48	1.5	4.27	0.78
E	Bondapak C_{18}	52/48	1.2	4.10	0.37
F	Bondapak C_{18}	52/48	1.5	4.25	0.83

where r and r' = responses for sample and std, resp.; C = concn of chlorpropamide std soln, mg/mL; W = sample wt, g; DF = diln factor for sample, mL; ATW = av. tablet wt, g/tab.

Results and Discussion

Collaborative results are presented in Tables 3 and 4. Detection wavelengths ranged from 232 to 240 nm. No problems were reported and all collaborators met the requirements for column performance.

The collaborative results were evaluated statistically by the general procedure described by Youden and Steiner (5). There were no outliers among individual or laboratory results by Dixon's test. No laboratory was deemed to be an outlier by the rank sum test. Homogeneity of experimental variation gave 0.41 for variation between laboratories (limiting value, 0.54) and 0.18 for variation between replicates (limiting value, 0.21).

The organic portion of the mobile phase must not be greater than 50% to ensure resolution of PCBS, which elutes before chlorpropamide. Chlorpropamide has an absorption maximum at 232 nm, but at this wavelength there is background absorption due to the presence of acetic acid, which may be difficult to correct, depending on available instrumentation. At the detection wavelength of 240 nm, this problem is eliminated. When values were plotted over the range 0.2–2.0 μg, the extrapolated line passed through the origin. Recoveries were quantitative over the range 0.85–1.15 μg.

At 240 nm, the absorptivity of PCBS is approximately 65% of that of chlorpropamide. The limit of detection for PCBS is 0.01 μg when 1 μg of chlorpropamide is injected. The other 2 potential impurities, propylamine and di-*n*-propylurea, do not absorb in the UV region and would not interfere in the determination of chlorpropamide.

The proposed LC method is specific and stability-indicating. The collaborative results show good accuracy and precision. Since the method is dependent neither on column nor exact wavelength, it is considered rugged. The detection wavelength should be specified as 240 nm to eliminate background correction problems and to impart method consistency.

Recommendation

It is recommended that the LC method for the determination of chlorpropamide in tablet dosage forms be adopted official first action.

Acknowledgments

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Emma Aranda, Los Angeles, CA
Miguel Colon, San Juan, PR

Table 4. Collaborative results by proposed method for chlorpropamide as % of theoretical or % of label declaration[a]

Coll.	Authentic (532.2 mg/g)	Lot 23 (250 mg/tab.)	Lot 90 (100 mg/tab.)
A	99.51, 99.09	98.16, 96.87	100.59, 100.02
B	99.61, 99.09	98.19, 99.36	101.14, 101.24
C	98.99, 99.51	98.05, 98.73	100.84, 100.73
D	99.44, 100.84	99.71, 100.32	101.87, 103.75
E	100.48, 100.14	98.52, 98.86	98.77, 101.76
F	99.18, 99.16	98.91, 101.67	101.76, 102.70
Mean	99.59	98.95	101.22
SD	0.60	1.23	1.26
RSD, %	0.60	1.24	1.24

[a]SD (repeatability) = 0.84; SD (reproducibility) = 1.10.

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Liquid Chromatographic Determination of Hydrazine in Polyvinylpyrrolidone

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A liquid chromatographic method for determination of hydrazine in polyvinylpyrrolidone (PVP) has been developed. After PVP is dissolved in acetate buffer, hydrazine is derivatized with benzaldehyde to form benzalazine, and is quantitated on a silica column using *p*-dinitrobenzene as the internal standard. The minimum quantitatable level of hydrazine is about 180 ppb, but with changes in the sampling procedure, this could readily be lowered to 90 ppb. Reproducibility on repeat analysis is about ± 10%. The method was used to analyze 16 lots of PVP from 3 commercial sources. Results ranged from nondetectable to 11 600 ppb; recoveries from spiked samples in most cases were 84% or better. Hydrazine levels tended to be lower in the higher molecular weight samples.

Polyvinylpyrrolidone (PVP) is used in the manufacture of pharmaceutical dosage forms and food, beverage, and cosmetic products where it may be ingested or come in contact with the skin, as well as in many other products. PVP is available in a range of molecular weight grades; molecular weight depends on the amounts of hydrogen peroxide and ammonia present during polymerization (1). Hydrazine may form as a by-product during polymerization, perhaps by a reaction similar to the Raschig process (2). Hydrazine is a mutagen (3) and a carcinogen (4) in laboratory animals.

Methods for the determination of hydrazine, as an azine derivative, at low levels in drugs (5) and other matrices (6) have been reported. These methods specify derivatization of hydrazine with an aldehyde followed by liquid chromatography. This paper describes a method for the determination of hydrazine in PVP, based on its reaction with benzaldehyde to form benzalazine, and reports the levels found in commercial materials. The USP monograph for PVP, as Povidone, does not specify a limit for hydrazine (7).

METHOD

Apparatus

(a) *Liquid chromatograph.*—Varian Model 5060, equipped with Vista 402 data processor, Model UV100 variable wavelength detector set at 295 nm, and Rheodyne Model 7125 injector fitted with 50 μL loop. Use 5 μm silica column (Resolve, Waters 150 × 3.9 mm or Lichrosorb SI_{60}, 250 × 4.6 mm) at ambient temperature with mobile phase flow rate of 1 mL/min.

(b) *Shaker.*—Horizontal type, Eberbach Corp. Mount reagent tubes on shaker by clips attached to board cut to fit shaker bed and attached to it.

Reagents

(a) *Solvents and reagents.*—Isopropyl alcohol, *n*-hexane (J. T. Baker Chemical Co., Phillipsburg, NJ), tetrahydrofuran (BDH Chemicals, Toronto, Ontario, Canada), and sodium acetate (Fisher Scientific Co., Fairlawn, NJ), LC grade. Benzaldehyde (Fisher Scientific Co., Fairlawn, NJ) and hydrazine sulfate (Sigma Chemical Co., St. Louis, MO), ACS Certified grade. *p*-Dinitrobenzene (J. T. Baker Chemical Co.).

(b) *Mobile phase.*—0.1% isopropyl alcohol and 2% tetrahydrofuran (v/v) in *n*-hexane.

(c) *Internal standard solution.*—0.010 mg/mL of *p*-dinitrobenzene in *n*-hexane. Dissolve ca 25 mg *p*-dinitrobenzene accurately weighed in 15 mL chloroform and dilute with *n*-hexane to 25 mL. Transfer 10.0 mL aliquot of this solution to 1 L volumetric flask and dilute to volume with *n*-hexane.

(d) *Buffer.*—0.05M sodium acetate in water, adjusted to pH 6.0 with 0.05M acetic acid.

(e) *Benzaldehyde reagent.*—25 mg/mL of benzaldehyde in methanol, prepared fresh daily. Store benzaldehyde under nitrogen after opening.

(f) *Standard solutions.*—Hydrazine sulfate in 0.05M sodium acetate buffer, prepared fresh daily: A: 1.35 μg/mL, B: 0.405 μg/mL, C: 0.135 μg/mL; and D: 0.0405 μg/mL.

Derivatization

To 3.0 mL aliquot of each standard solution in separate 125 × 16 mm culture tubes fitted with Teflon-lined screw caps, add 1.0 mL benzaldehyde reagent solution and shake vigorously 45 min on flat-bed shaker. Add 15.0 mL internal standard solution and continue shaking 30 min. Centrifuge at

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Table 1. Hydrazine levels in polyvinylpyrrolidone[a]

Sample	Manuf.	K-Value	Hydrazine level, ppb	Recovery from spiked samples, %[b]
1	A	17	11600[c]	60
2	A	30	346[d]	98
3	A	25	463; 462	94
4	A	25	255; 296	90
5	A	90	none detected[e]	92
6	B	44 000[f]	315; 355	91
7	B	700 000[f]	778; 819	84
8	C	29–32	1300; 1700	98
9	C	29–32	163; 196	87
10	C	17	6800[g]	87
11	C	24	784; 720; 830	88
12	C	26–28	382; 432	95
13	C	30	1000; 1100; 1100	92
14	C	26–28	900; 1000	92
15	C	30	133; 240; 241	93
16	C	90	none detected[e]	97

[a]Each determination is the average of duplicate injections.
[b]Recovery of hydrazine from spiked samples. The amount spiked was equivalent to 850 ppb hydrazine.
[c]Mean of 9 determinations; CV = 7.3%
[d]Mean of 5 determinations; CV = 4.2%
[e]Minimum detectable level based on 200 mg sample (666.6 μg PVP on column is 45 ppb).
[f]Actual molecular weights. The labels on these products do not specify whether these are number, weight, or viscosity average molecular weights.
[g]Mean of 4 determinations; CV = 12.7%

3500 rpm for 1 min and immediately separate a portion of organic phase from aqueous phase.

System Suitability

(a) Condition column until baseline is stable (ca 1 h). Inject five 50 μL aliquots of organic phase after derivatization of standard solution B. In suitable system, resolution of benzalazine and benzaldehyde peaks is > 1.0, and relative standard deviation of ratios of benzalazine to internal standard peak responses is < 1%.

(b) Inject duplicate 50 μL aliquots of organic phase of each derivatized standard solution and record peak responses. Calculate response factor for each standard solution by C_1W_2/C_2W_1, where C_1 and C_2 = responses of benzalazine and *p*-dinitrobenzene peaks, respectively, and W_1 and W_2 = concentration in μg/mL of hydrazine sulfate in standard solution and of *p*-dinitrobenzene in internal standard solution, respectively. In suitable system, relative standard deviation of response factors over concentration range of standard solutions is < 10%.

PVP Samples

K-Values as listed in Table 1 are expressions of molecular weights and are defined in USP monograph for Povidone (7). *K*-Values of 17, 25, 30, and 90 correspond to average molecular weights of about 9500, 27000, 49000, and 1.1 million, respectively (8).

Procedure

Accurately weigh ca 100 mg polyvinylpyrrolidone into 125 × 16 mm culture tube equipped with Teflon-lined screw cap. Add 3.0 mL 0.05M sodium acetate buffer and shake to dissolve. Add 1.0 mL benzaldehyde reagent and shake vigorously 45 min on flat-bed shaker, add 15.0 mL internal standard solution, and continue shaking 30 min. Centrifuge at 3500 rpm for 1 min and immediately separate portion of organic phase from aqueous phase. Inject duplicate 50 μL aliquots of upper organic phase and record peak responses. If area of benzalazine peak in sample exceeds that of most concentrated calibration standard, repeat assay using appropriately smaller sample weight. Calculate weight in ng of hydrazine in 1 g polyvinylpyrrolidone by $(3 \times 10^6)(32.05/130.12)(C/W)(R_u/R_s)$, where 32.05 and 130.12 = molecular weights of hydrazine and hydrazine sulfate, respectively, C = concentration in μg/mL of hydrazine sulfate in standard solution, W = weight in mg of polyvinylpyrrolidone sample taken, and R_u and R_s = response ratios of benzalazine to *p*-dinitrobenzene peaks obtained from sample preparation and standard solution, respectively. For C and R_s, select standard solution closest to sample response.

Results and Discussion

The dependence of the derivatization reaction on time was determined in solutions containing about 35 mg/mL of PVP K90 and 0.2647 μg/mL of hydrazine sulfate. For reaction times from 5 to 60 min, recoveries ranged from 83 to 100%, with complete reaction after 30 min. A reaction time of 45 min is specified in the method. Concentrations of PVP K90 up to 100 mg/mL did not affect the extent of derivatization at a 45 min reaction time; the method specifies a PVP concentration of about 35 mg/mL.

Recoveries of hydrazine as benzalazine in the presence of 35 mg/mL of PVP K90 were determined for hydrazine sulfate levels from 0.05 to 2.00 μg/mL. Over this range, recoveries averaged about 89% and were independent of the original concentrations of hydrazine sulfate. All other conditions were the same as described in the method. Similarly, benzaldehyde was varied from 5 to 25 mg/mL; recoveries ranged from about 80 to 89%. The method calls for a benzaldehyde concentration of 25 mg/mL. The effect of PVP concentrations from 33 to 165 mg/mL on the extraction of benzalazine into *n*-hexane was determined on solutions containing 0.49 and 25 mg/mL of benzalazine and benzaldehyde, respectively. Recoveries were virtually complete over the entire range of PVP concentrations examined.

The system response was linear over a range from 0.12 to 4.92 ng of hydrazine, as benzalazine, on column, corresponding to 300 to 15 000 ppb hydrazine in PVP. The mean ratio (5 points) of the peak areas of benzalazine to internal standard to the corresponding weight ratio was 5.15 with a relative standard deviation of 4.5%.

The minimum amount of hydrazine that can be quantitated on column, as benzalazine, is 0.06 ng, corresponding to about

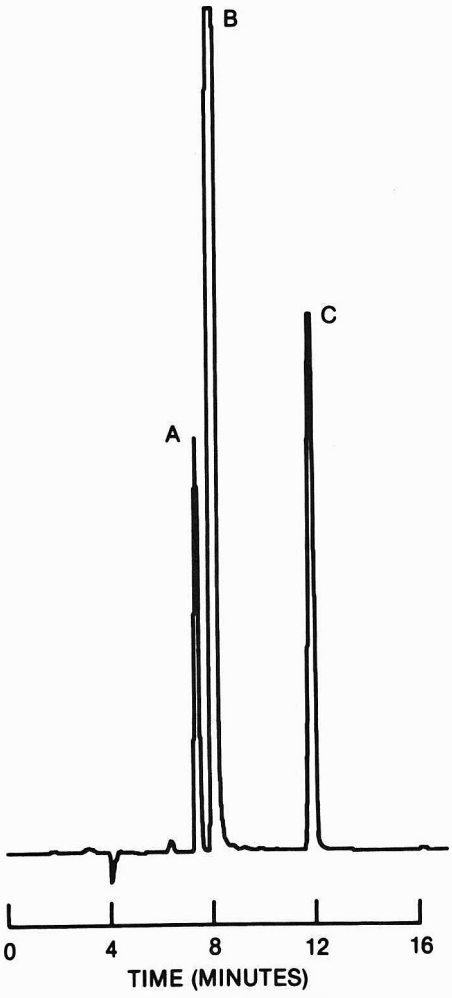

Figure 1. Chromatogram showing resolution of benzalazine (peak A), benzaldehyde (peak B), and *p*-dinitrobenzene internal standard (peak C) on 250 mm silica column. Amount of benzalazine on column was equivalent to 1.7 ng hydrazine base. Integrator was attenuated at 8, equivalent to detector response of 0.02 AUFS.

180 ppb in PVP. If necessary, sensitivity can be doubled by doubling the amount of PVP taken for analysis.

The development of this method was done using a 150 mm column. Typical retention times for benzalazine, benzaldehyde, and *p*-dinitrobenzene were 2.75, 2.90, and 3.90 min, respectively. The method was checked using a 250 mm column (Lichrosorb SI-60); a typical chromatogram obtained with this column is shown in Figure 1. Sixteen samples of various molecular weight grades of PVP from 3 commercial sources were analyzed for hydrazine (Table 1). Hydrazine levels were highest in the low molecular weight products; samples 1 and 10 showed levels well in excess of 1 ppm while none was detected in high molecular weight samples 5 and 16.

The identity of hydrazine in Sample No. 1 (Table 1) was established by comparison of the mass spectrum of the benzaldehyde derivative to that of an authentic sample of benzalazine.

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DECOMPOSITION IN FOODS

Headspace Gas Chromatographic Method for Determination of Ethanol in Canned Salmon: Collaborative Study

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Six laboratories collaboratively studied a headspace gas chromatographic method for determination of ethanol in the aqueous phase of canned salmon. Ethanol is determined by a headspace sampling technique with *tert*-butanol as the internal standard, using a gas chromatograph equipped with a Super Q column and a flame ionization detector. With outliers excluded, the mean recoveries from samples spiked with 25.1 and 78.4 ppm ethanol were 112 and 110%, respectively. For the 4 sample pairs quantitated, repeatability coefficients of variation ranged from 1.42 to 4.25% and reproducibility coefficients of variation from 2.55 to 8.09%, with 3 of the 4 reported values less than 5%. The method has been adopted official first action.

Results of previous work indicated a correlation between the ethanol content of the aqueous phase of canned salmon and the sensory classification of decomposition, and suggested that this relationship could be used to confirm an initial sensory classification (1). A simple, rapid headspace gas chromatographic method was developed for the determination of ethanol in the aqueous phase of canned salmon (2).

Because of the excellent results obtained with this method and its practicality, a collaborative study was conducted. The results of that study are described here.

Collaborative Study

Each of the 6 collaborators received samples of frozen canned salmon aqueous phase packed in dry ice in an insulated container. These samples consisted of a practice sample and 5 unknown sample pairs (Table 1). Each sample pair consisted of blind duplicates of the same sample. In addition, each collaborator received a set of instructions and a copy of the method.

Ethanol in Canned Salmon

Headspace Gas Chromatographic Method

First Action

18.B01 ***Principle***

Liq. from canned salmon is sepd into oil and aq. phases. Aq. phase is analyzed for EtOH by headspace gas chromatgy and flame ionization detection. Ratios of peak areas of EtOH to internal std in sample and std are compared.

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This report of the Associate Referee, H. R. Throm, was presented by T. A. Hollingworth, Jr, at the 99th AOAC Annual International Meeting, Oct. 27–31, 1985, at Washington, DC.
The recommendation of the Associate Referee was approved by the General Referee and the Committee on Foods I and was adopted by the Association. See the General Referee and Committee reports, *J. Assoc. Off. Anal. Chem.* (1986) 69, March issue.

18.B02 ***Apparatus***

(a) *Gas chromatograph.*—Model 5880A, equipped with flame ionization detector (Hewlett-Packard, Avondale, PA), or equiv. (Equiv. system must include electronic data system or integrator capable of measuring peak areas for off-scale peaks.) Representative operating conditions: temps—injector 200°, detector 250°, column 150°; gas flows—N carrier gas, 50 mL/min, H 45 mL/min, air 500 mL/min. Suggested sensitivity: Choose attenuation so that injection of 5 mL headspace from ca 11 ppm headspace std (contains ca 11 ppm EtOH and 4.2 ppm *tert*-BuOH; see prepn of headspace stds) gives *tert*-BuOH peak ≥50% <100% FSD. EtOH peak will then be ≥25% FSD.

(b) *Gas chromatographic column.*—6 ft × 4 mm id glass, packed with 80–100 mesh Super Q (Alltech Associates, Inc., Deerfield, IL). With column in place and connected to detector, purge with N carrier gas at 50 mL/min at 33° (ambient temp.) for 30 min. Increase temp. at 3°/min to 225° and hold for 2 h. Decrease to operating temp., let column stabilize, adjust carrier gas flow to 50 mL/min, and let column further stabilize overnight.

System check.—For operating conditions given in (a), retention times are ca 3.5–3.8 min for EtOH; and ca 10.4–11.5 min for *tert*-BuOH. However, adjust column temp. for adequate resolution between EtOH and *tert*-BuOH peaks, and any significant product peaks. For some products, small peak may occur at retention time ca 8.4–8.8 min; this should not significantly overlap *tert*-BuOH peak.

(c) *Syringes.*—Gas-tight, Hamilton No. 1005-LTN (5.0 mL capacity) or No. 1010-LTN (10.0 mL capacity) (The Anspec Co., Inc., Ann Arbor, MI).

(d) *Headspace vials.*—Glass, Kimble Cat. No. 60910-L, 23 × 85 mm, 6 dram (ca 22 mL) capacity (Ace Glass, Inc.) fitted with perforated screw cap (Cat. No. 95053) with Teflon-faced liner (Cat. No. 9522) (both screw cap and liner from Alltech Associates, Inc.).

(e) *Continuously adjustable digital microliter pipet.*—Pipetman Model P-200, 20–200 μL range (Gilson, Cat. No. P-200), equipped with disposable microliter pipet tips (Rainin Cat. No. RC-20) (both from Rainin Instrument Co., Inc., Woburn, MA), or equiv.

18.B03 ***Reagents***

(a) *Sodium chloride crystal.*—Baker Analyzed Reagent (J.T. Baker Chemical Co.).

(b) *tert-Butanol internal std solns.*—*(1) Stock soln.*—Approx. 6000 ppm. Into tared 250 mL vol. flask, pipet 2.0 mL liq. *tert*-BuOH (99.5%, Aldrich Chemical Co., Inc.). Reweigh stoppered flask and its contents to det. wt of *tert*-BuOH (ca 1.50 g). Dil. to vol. with H_2O. *(2) Working soln.*—Approx. 108 ppm. Pipet 9.0 mL stock soln into 500 mL vol. flask and dil. to vol. with H_2O.

(c) *Ethanol std solns.*—*(1) Stock soln.*—Approx. 15 600 ppm. Into tared 100 mL vol. flask, pipet 2.0 mL absolute alcohol (USP, U.S. Industrial Chemicals Co., New York, NY). Reweigh stoppered

Table 1. Collaborative results for determination of ethanol in canned salmon aqueous phase as blind duplicate samples

	Ethanol found						
			Sample 3		Sample 4		
Coll.	Sample 1, ppm	Sample 2, ppm	ppm	Rec., %	ppm	Rec., %	Sample 5, ppm
Present:	blank	~14[a]	25.1[b]		78.4[b]		~100[a]
1	0	14.0	29.5	117.5	90.0	114.8	125.0[c]
	0	15.0	27.5	109.6	86.5	110.3	109.0[c]
2	0	14.0	27.5	109.6	85.0	108.4	108.0
	0	14.5	27.5	109.6	85.5	109.1	107.0
3	0	15.5	28.5	113.5	85.0	108.4	108.0
	0	16.0	27.5	109.6	86.0	109.7	103.0
4	0	16.0	26.0	103.6	86.0	109.7	100.5
	0	14.5	28.0	111.6	84.5	107.8	105.0
5	0	13.0	25.0[d]	99.6	83.0	105.9	109.0
	0	13.0	35.5[d]	141.4	84.5	107.8	109.5
6	0	16.5	29.0	115.5	89.0	113.5	100.5
	0	15.5	29.5	117.5	89.0	113.5	105.0

[a]Naturally occurring.
[b]Spiked.
[c]Dixon and Cochran outlier.
[d]Cochran outlier.

flask and its contents to det. wt of EtOH (ca 1.56 g). Dil. to vol. with H_2O. (*2*) *Intermediate soln.*—Approx. 1090 ppm. Pipet 7.0 mL stock soln into 100 mL vol. flask and dil. to vol. with H_2O. (*3*) *Working solns.*—Solns A, B, C, and D, ca 22, 44, 76, and 109 ppm, resp. Pipet 2.0, 4.0, 7.0, and 10.0 mL intermediate soln into sep. 100 mL vol. flasks and dil. each to vol. with H_2O. Soln E, ca 11 ppm. Pipet 10.0 mL soln D (ca 109 ppm) into 100 mL vol. flask and dil. to vol. with H_2O.

Prep. fresh EtOH std solns weekly.

18.B04 *Check for Possible Contamination*

(**a**) *Air.*—Using gas-tight syringe, inject 5 mL air (in location where aliquots will be withdrawn from headspace stds and samples) into GC app. to ensure that syringe is not contaminated and that air does not contain compds that significantly interfere with EtOH and *tert*-BuOH peaks in analysis.

(**b**) *Blank.*—Pipet 5.0 mL H_2O into glass headspace vial. Add 3.0 g NaCl and seal. Hold vial in vertical position so as not to wet Teflon-faced liner while swirling. Swirl contents vigorously 2 min, and then let stand ≥5 min. Withdraw 5 mL aliquot from headspace into gas-tight syringe by withdrawing plunger in single, slow, continuous action; then inject it into the GC app. to det. any significant interference with EtOH and *tert*-BuOH peaks in analysis. This should be done in same location where air is analyzed for contamination, (**a**).

When air contains low levels of interfering compds, analysis of blank may reveal that levels are too low to cause significant interference with headspace analysis. However, analysis of blank may indicate that headspace must be withdrawn from headspace vials where air is free from interfering compds. If air in room is contaminated with EtOH, headspace stds and samples can be prepd in that room, but headspace must be withdrawn in area free of EtOH contamination. If it is then brought back into contaminated room and immediately injected into GC app., analysis will not be contaminated with EtOH.

18.B05 *Preparation of Headspace Standards*

Pipet 5.0 mL EtOH working std soln into glass headspace vial. Then add 200 μL *tert*-BuOH working internal std soln (ca 108 ppm), using adjustable microliter pipet (Model P-200) to give *tert*-BuOH concn of ca 4.2 ppm. Gently mix 3–4 s, add 3.0 g NaCl, and seal vial. Holding vial in vertical position so as not to wet Teflon-faced liner while swirling, swirl contents vigorously 2 min, and then let stand ≥5 min. Inject ca 5 mL aliquot of headspace into GC app. (see below).

18.B06 *Preparation of Calibration Curve*

Prep. headspace stds as previously described using EtOH working std solns A, B, C, D, and E (ca 11–109 ppm). Analyze each headspace std as follows: Withdraw 5 mL aliquot of headspace from vial into gas-tight syringe by withdrawing plunger in single, slow, continuous action; then inject it into GC app. If for any reason a second injection is required, prep. new headspace std. Between injections, pump syringe ≥10–15 times to eliminate gases and H_2O vapor from previous injection to avoid contamination.

Use peak areas only for quantitation. To det. peak area, use tangent skimming for EtOH peak, on tail of air peak. For each headspace std, calc. peak area ratio, R = area EtOH peak/area *tert*-BuOH peak. Prep. calibration curve as follows: For each headspace std, plot R against concn of EtOH working std soln used to prep. that std. Draw best curve that fits points on graph, or use automated curve fitting or multi-level calibration if instrument is so equipped.

18.B07 *Canned Salmon Aqueous Phase*

Open can and drain liq. into 250 mL beaker while pressing lid against contents. Retain salmon for sensory analysis if appropriate. Transfer liq. to 250 mL separator and let oil and aq. phases sep. Drain aq. phase into g-s cylinder and store until analysis.

18.B08 *Preparation of Headspace Samples*

Use same procedure given for prepn of headspace stds, except transfer 5.0 mL sample soln into headspace vial with accurate 5 mL Mohr-style pipet. For sample soln, use either undild canned salmon aq. phase, or, when necessary for GC analysis (see below), canned salmon aq. phase accurately dild with H_2O.

18.B09 *Analysis of Headspace Samples*

Use same procedure given for analysis of headspace stds (see prepn of calibration curve) and inject 5 mL aliquot of headspace into GC app. Analysis time for samples is ca 38 min because of late-eluting peak at ca 35 min.

Calc. peak area ratio, R, and det. EtOH concn in sample soln from calibration curve.

If EtOH concn is higher than that of most concd std, accurately dil. original canned salmon aq. phase with H_2O to give concn within calibration limits, and reanalyze dild sample. Multiply by diln factor to obtain concn for original undild sample.

Table 2. Statistical analysis of collaborative study data[a]

Statistic	Sample 2	Sample 3	Sample 4	Sample 5
		Outliers included		
Mean				
Found, ppm	14.79	28.38	86.17	107.46
Rec., %	—	113.07	109.91	—
Repeatability				
SD	0.63	3.02	1.22	5.19
CV, %	4.25	10.63	1.42	4.83
Reproducibility				
SD	1.20	3.02	2.19	6.48
CV, %	8.09	10.63	2.55	6.03
		Outliers excluded		
Mean				
Found, ppm	14.79	28.05	86.17	105.55
Rec., %	—	111.75	109.91	—
Repeatability				
SD	0.63	0.96	1.22	2.58
CV, %	4.25	3.43	1.42	2.45
Reproducibility				
SD	1.20	1.11	2.19	3.41
CV, %	8.09	3.95	2.55	3.23
Outlying results,				
Coll. No.		5		1

[a]Sample 1 (blank) not included in data analysis.

Results and Discussion

The results obtained by the 6 collaborating laboratories are summarized in Table 1. In addition to the ethanol concentration (ppm) determined by the collaborators for all samples, Table 1 lists the percent recoveries obtained for the samples spiked with 25.1 and 78.4 ppm ethanol, samples 3 and 4, respectively. The data (with the exception of sample 1, which was not included in the data analysis because it was the blank) were checked for outliers by the Youden rank sum test, the Dixon test, and the Cochran test (3). No outliers were found by the Youden rank sum test, but 1 sample was detected as a Cochran outlier (sample 3, Collaborator 5) and 1 sample was detected as both a Dixon and a Cochran outlier (sample 5, Collaborator 1).

The data (Table 1) were statistically analyzed and the results were summarized (Table 2). The mean values, as well as the repeatability and reproducibility standard deviations (SD) and their corresponding coefficients of variation (CV), were calculated with and without the specified outliers. With outliers included, the repeatability CVs ranged from 1.42 to 10.63% and the reproducibility CVs from 2.55 to 10.63% (Table 2). With outliers excluded, the repeatability CVs varied from 1.42 to 4.25% and the reproducibility CVs from 2.55 to 8.09% (Table 2). Exclusion of the 2 outliers reduced the coefficients of variation by at least 45%. With the exclusion of these outliers, the repeatability CV for each of the 4 samples is less than 5%. The same is true for the reproducibility CVs, with the exception of sample 2 (8.09%).

The collaborating laboratories used various models of gas chromatographs to analyze the collaborative study samples. None of the collaborators reported problems with the method.

In conclusion, this collaborative study describes a method for the determination of ethanol in canned salmon with good repeatability and reproducibility and with few outliers. This method should be useful to confirm the initial sensory classification of decomposition of the product.

Recommendation

It is recommended that the headspace gas chromatographic method for the determination of ethanol in canned salmon be adopted official first action.

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F. Hons, Food and Drug Administration (FDA), Dallas, TX

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MICROBIOLOGICAL METHODS

Enumeration of Total Bacteria and Coliforms in Milk by Dry Rehydratable Film Methods: Collaborative Study

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Eleven laboratories participated in a collaborative study to compare the dry rehydratable film (Petrifilm® SM and Petrifilm® VRB) methods, respectively, to the standard plate count (SPC) and violet red bile agar (VRBA) standard methods for estimation of total bacteria and coliform counts in raw and homogenized pasteurized milk. Each laboratory analyzed 16 samples (8 different samples in blind duplicate) for total count by both the SPC and Petrifilm SM methods. A second set of 16 samples was analyzed by the VRBA and Petrifilm VRB methods. The repeatability standard deviations (the square root of the between-replicates variance) of the SPC, Petrifilm SM, VRBA, and Petrifilm VRB methods were 0.05104, 0.0444, 0.14606, and 0.13806, respectively; the reproducibility standard deviations were 0.7197, 0.06380, 0.15326, and 0.13806, respectively. The difference between the mean $\log_{10}$ SPC and the mean $\log_{10}$ Petrifilm SM results was 0.027. For the VRBA and Petrifilm VRB methods, the mean $\log_{10}$ difference was 0.013. These results generally indicate the suitability of the dry rehydratable film methods as alternatives to the SPC and VRBA methods for milk samples. The methods have been adopted official first action.

The standard plate count (SPC) method (1) is recognized as the standard method for enumerating total bacteria count in raw and pasteurized milk. Violet red bile agar (VRBA) (2) is the standard solid medium used in the enumeration of coliform organisms in raw and pasteurized milk. The Petrifilm® SM and Petrifilm® VRB methods were developed as alternatives to the SPC and VRBA methods, respectively. Both of these dry rehydratable film methods have undergone successful within-laboratory comparative investigation (2, 3). Both have certain inherent advantages over the standard methods. Petrifilm is available as self-contained culture plates of dry media to which milk samples (in full strength or as dilutions) can be added directly. No sterilization step is required; no pouring of plates is necessary. The Petrifilm plates accept samples by pipet or by plate loop continuous pipetting syringe (1).

Collaborative Study

Eleven laboratories served as collaborators in this study. Each collaborator received a complete set of instructions, data sheets, and test materials. Milk samples analyzed by the collaborators were prepared and distributed in the following manner: Four days before distribution, stock supplies of raw and pasteurized homogenized milk were obtained by the Dairy Quality Control Institute, Inc. The raw milk was sterilized by heating in an autoclave for 18 min at 121°C. The homogenized pasteurized milk was also sterilized in an autoclave, but for 13 min at 121°C. After autoclaving, stock supplies were cooled in ice and then stored at 4°C.

On the day of distribution, both stock milk supplies were inoculated with mixed cultures obtained from the Minnesota Department of Agriculture. These cultures represented those groups of organisms utilized in the Split Milk Program for certification of Minnesota dairy laboratories.

Mixtures of *Escherichia coli* and *Staphylococcus aureus* were prepared in 2 concentrations (approximately 2500–3000 and 1000–1500 organisms/mL). Similar mixtures of *E. coli* and *Streptococcus lactis* were also prepared. These 4 mixtures were used as such (with 1 dilution) in making total count determinations and, at a 10-fold reduction in cfu/mL, for coliform determinations. All stock cultures, therefore, consisted of both gram-negative and gram-positive organisms.

From these sources, 16 samples (8 samples in blind duplicate) each for total and coliform enumeration, were dispensed into plastic vials for shipment to collaborating laboratories. Test samples for total bacteria count determination were inoculated at bacterial levels necessitating a 1 to 10 dilution. Collaborators were provided with Petrifilm SM and Petrifilm VRB plates, control lots of SMA and VRBA, and 11 mL pipets for making the 1 to 10 dilution of total count samples. All samples were handled and analyses were performed according to techniques prescribed and/or updated in *Standard Methods for the Examination of Dairy Products* (1).

Bacterial and Coliform Counts in Milk
Dry Rehydratable Film Methods
First Action

46.B05 ***Principle***

Method uses bacterial culture plates of dry medium and cold H_2O-sol. gel. Undild or dild samples are added directly to plates at rate of 1.0 mL per plate. Pressure, when applied to plastic spreader placed on overlay film, spreads sample over ca 20 sq. cm growth area. Gelling agent is allowed to solidify and plates are incubated and then counted. Either pipet or plate loop continuous pipetting syringe can be used for sample addn for bacterial count analyses.

46.B06 ***Apparatus***

(a) *Std method plates.*—Plates contain std methods media nutrients, **46.005(g)**, cold H_2O-sol. gelling agent coated onto film base,

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This report of the Associate Referees, R. E. Ginn and V. S. Packard, was presented at the 99th AOAC Annual International Meeting, Oct. 27–31, 1985, at Washington, DC.

Table 1. Actual counts/mL of blind duplicates of 8 milk samples, by 11 collaborators using Petrifilm SM (PSM) and standard plate count (SPC) methods

	1		2		3		4		5		6		7		8	
Lab.	PSM	SPC	PSM	SPC	PSM	SPC	PSM	SPC	PSM	SPC	PSM	SPC	PSM	SPC	PSM	SPC
1	5500	5430	1500	1610	3580	3780	1510	1600	4260	3850	1450	1520	3420	3370	1990	1690
	5390	5130	1650	1540	3460	3270	1590	1610	3910	4490	1610	1560	3280	3060	2010	2060
2	4600	4560	1610	1520	3200	2850	1560	1200	3600	3990	1590	1470	3410	2580	1980	1480
	4400	3990	1350	1360	3420	2900	1570	1210	3280	2890	1510	1510	2910	2840	1880	1600
3	4760	5200	1560	1280	3340	3320	1630	1610	3440	3270	1540	1580	2910	3590	1780	1870
	5280	4380	1650	1570	4600	3100	1600	1560	3720	3630	1910	1620	3010	3650	1670	1540
4	4600	4600	1350	1680	3080	3000	1760	1670	3580	2860	1690	1370	5000	5400	1840	1880
	4950	4800	1240	1320	2960	3160	1620	1420	3500	3220	1610	1320	2580	2560	1750	1950
5	5700	4100	1400	880	2040	2600	1400	1100	3800	3000	1600	1300	2700	2400	1700	1400
	4400	3900	1500	1200	2300	2300	1600	1000	3500	2700	1700	1400	2700	2300	1800	1100
6	4000	3500	1800	1700	2800	3000	1600	1600	3000	2800	1900	1900	2300	2700	1700	1600
	3800	4400	1500	1400	4400	4800	1300	1300	3900	4100	1600	1500	3000	3100	1800	1700
7	5700	5800	1700	1400	4000	3800	1600	1500	4400	4600	2100	1600	3320	3000	2100	1700
	6200	5900	1700	1600	3700	3800	1600	1600	4500	5400	2000	1600	2900	3000	2000	1800
8	4980	4740	1950	1560	2240	3050	1790	1390	3700	3290	2200	1560	2550	2520	1970	1840
	5320	4480	1910	1410	2620	2850	1570	1340	3920	3100	2060	1840	2070	2880	1730	1620
9	4310	4430	1370	1330	2890	2770	1420	1630	3390	3330	1480	1300	2910	2600	1630	1690
	4150	4800	1260	1390	3130	3720	1440	1310	3320	3050	1650	1630	2640	2780	1900	1760
10	3440	3960	1480	1520	2840	3110	1280	1540	2890	3290	1370	1230	3080	2960	1900	1800
	5000	4130	1510	1330	3050	3220	1560	1450	3060	2980	1500	1510	3020	2720	1670	1570
11	3750	3620	1300	1210	2630	2390	1350	1180	2940	3030	1370	1150	2380	1730	1530	1390
	4350	3630	1340	1120	2350	2510	1500	1130	2910	2870	1700	1200	2350	2530	1620	1260

Table 2. Actual counts/mL of blind duplicates of 8 milk samples by 11 collaborators using Petrifilm VRB (PET) and standard violet red bile agar (STD) methods

	1		2		3		4		5		6		7		8	
Lab.	PET	STD	PET	STD	PET	STD	PET	STD	PET	STD	PET	STD	PET	STD	PET	STD
1	51	46	20	16	49	45	21	18	1	5	21	17	18	17	16	17
	57	44	11	11	44	43	21	17	1	2	18	10	25	25	11	17
2	47	55	14	15	43	47	13	18	2	1	16	15	28	26	18	17
	43	48	15	16	42	33	18	25	1	4	18	18	31	27	13	11
3	33	37	4	13	43	32	21	23	5	1	15	11	27	31	9	13
	37	59	18	15	47	43	21	23	4	4	16	11	27	32	13	11
4	46	42	21	11	38	41	24	24	5	3	23	11	23	15	13	12
	43	39	8	7	52	37	22	20	1	4	27	10	28	30	11	7
5	41	31	11	13	39	31	20	15	5	2	18	13	25	21	13	20
	41	31	9	9	35	44	19	21	2	1	12	13	26	19	10	7
6	45	47	16	10	42	50	30	26	3	6	17	17	15	21	16	10
	43	43	7	14	40	37	17	22	3	1	11	18	24	23	7	11
7	50	46	12	14	64	56	32	26	4	8	14	16	26	30	8	12
	50	54	12	12	50	42	30	20	2	4	16	16	36	38	16	10
8	41	48	14	14	39	38	21	23	3	5	9	14	34	23	16	15
	35	52	15	13	41	56	19	20	2	3	13	17	17	11	9	9
9	39	59	10	18	43	51	19	30	6	2	16	17	25	36	9	16
	41	50	10	11	61	40	22	29	1	4	13	14	27	21	15	16
10	39	36	12	18	43	62	22	28	4	1	17	24	28	26	10	12
	47	56	11	9	43	54	21	26	3	5	18	22	21	24	15	14
11	50	53	19	17	33	45	22	25	3	5	22	22	22	35	13	14
	51	45	12	12	38	46	25	19	2	3	14	16	20	26	13	18

overlay film coated with gelling agent, and 2,3,5-triphenyltetrazolium chloride indicator. Circular growth area of single plate contains ca twenty 1 cm squares outlined on film base. Petrifilm SM Plates® (available from Medical-Surgical Division/3M, 225-5S 3M Center, St. Paul, MN 55144) or equiv. meets these specifications.

(b) *Violet red bile plates.*—Plates contain violet red bile nutrients conforming to APHA standards as given in Compendium of Methods for the Microbiological Examination of Foods, 2nd ed., 1984 (American Public Health Association, 1015 18th St, NW, Washington, DC 20005), cold H_2O sol. gelling agent, and 2,3,5-triphenyltetrazolium chloride. Petrifilm VRB Plates® (available from Medical-Surgical Division/3M), or equiv. meets these specifications.

(c) *Plastic spreader.*—Provided with Petrifilm plates, consists of concave side and smooth flat side, designed to spread milk sample evenly over plate growth area.

(d) *Pipets.*—Calibrated for bacteriological use or plate loop continuous pipetting syringe to deliver 1.0 mL.

(e) *Colony counter*—Std app., Quebec model preferred, or one providing equiv. magnification and visibility.

46.B07 ***Analysis***

(a) *Bacterial colony count.*—Use Petrifilm SM or equiv. plates. Place plate on flat surface. Lift top film and inoculate 1 mL sample

Table 3. Mean $\log_{10}$ counts/mL of blind duplicates of 8 milk samples by 11 collaborators using Petrifilm SM (PSM) and standard plate count (SPC) methods

	1		2		3		4		5		6		7		8	
Lab.	PSM	SPC	PSM	SPC	PSM	SPC	PSM	SPC	PSM	SPC	PSM	SPC	PSM	SPC	PSM	SPC
1	3.736	3.722	3.197	3.197	3.546	3.546	3.190	3.205	3.611	3.619	3.184	3.187	3.525	3.507	3.301	3.271
2	3.653	3.630	3.169	3.158	3.520	3.459	3.195	3.081	3.536	3.531	3.190	3.173	3.498	3.432	3.285	3.187
3	3.700	3.679	3.205	3.152	3.593	3.506	3.208	3.200	3.554	3.537	3.234	3.204	3.471	3.559	3.237	3.230
4	3.679	3.672	3.112	3.173	3.480	3.488	3.228	3.188	3.549	3.482	3.217	3.129	3.555	3.570	3.254	3.282
5	3.700	3.602	3.161	3.012	3.336	3.388	3.175	3.021	3.562	3.454	3.217	3.130	3.431	3.371	3.243	3.094
6	3.591	3.594	3.216	3.188	3.545	3.579	3.159	3.159	3.534	3.530	3.241	3.227	3.419	3.461	3.243	3.217
7	3.774	3.767	3.230	3.175	3.585	3.580	3.204	3.190	3.648	3.698	3.312	3.204	3.492	3.477	3.312	3.243
8	3.712	3.664	3.286	3.171	3.384	3.470	3.224	3.135	3.581	3.504	3.328	3.229	3.361	3.430	3.266	3.237
9	3.626	3.664	3.119	3.133	3.478	3.507	3.155	3.165	3.526	3.503	3.194	3.163	3.443	3.430	3.245	3.237
10	3.618	3.607	3.175	3.153	3.469	3.500	3.150	3.174	3.473	3.496	3.156	3.134	3.484	3.453	3.251	3.226
11	3.606	3.559	3.121	3.066	3.396	3.389	3.153	3.062	3.466	3.470	3.184	3.070	3.374	3.321	3.197	3.122
Mean	3.672	3.651	3.181	3.143	3.485	3.492	3.186	3.144	3.549	3.529	3.223	3.168	3.459	3.456	3.258	3.213

Overall mean (all samples, all laboratories) SPC = 3.350
Overall mean (all samples, all laboratories) Petrifilm SM = 3.377
difference = 0.027

Table 4. Mean $\log_{10}$ coliform counts/mL of blind duplicates of 8 milk samples by 11 collaborators using Petrifilm VRB (PET) and standard violet red bile agar (STD) methods

	1		2		3		4		5		6		7		8	
Lab.	PET	STD	PET	STD	PET	STD	PET	STD	PET	STD	PET	STD	PET	STD	PET	STD
1	1.732	1.653	1.171	1.123	1.667	1.643	1.322	1.243	0.000	0.500	1.289	1.115	1.327	1.314	1.123	1.230
2	1.653	1.711	1.161	1.190	1.628	1.595	1.185	1.327	0.151	0.301	1.230	1.216	1.469	1.423	1.185	1.136
3	1.543	1.670	0.929	1.145	1.653	1.569	1.322	1.362	0.651	0.301	1.190	1.041	1.431	1.498	1.034	1.078
4	1.648	1.607	1.113	0.943	1.648	1.590	1.361	1.341	0.349	0.540	1.397	1.021	1.404	1.327	1.078	0.962
5	1.613	1.491	0.998	1.034	1.568	1.567	1.290	1.249	0.500	0.151	1.167	1.114	1.406	1.300	1.057	1.073
6	1.643	1.653	1.025	1.073	1.613	1.634	1.354	1.379	0.477	0.389	1.136	1.243	1.278	1.342	1.025	1.021
7	1.699	1.698	1.079	1.113	1.753	1.686	1.491	1.358	0.452	0.753	1.175	1.204	1.486	1.528	1.054	1.040
8	1.578	1.699	1.161	1.130	1.602	1.664	1.300	1.331	0.389	0.588	1.034	1.188	1.381	1.202	1.079	1.065
9	1.602	1.735	1.000	1.148	1.709	1.655	1.311	1.470	0.389	0.452	1.159	1.188	1.415	1.439	1.065	1.204
10	1.632	1.652	1.060	1.105	1.633	1.762	1.332	1.431	0.540	0.349	1.243	1.361	1.385	1.398	1.088	1.113
11	1.703	1.689	1.179	1.155	1.549	1.658	1.370	1.338	0.389	0.588	1.244	1.273	1.322	1.480	1.114	1.201
Mean	1.641	1.660	1.080	1.105	1.638	1.639	1.331	1.348	0.390	0.446	1.206	1.179	1.391	1.386	1.082	1.102

Overall mean (all samples, all laboratories) standard VRBA = 1.233
Overall mean (all samples, all laboratories) Petrifilm VRB = 1.220
difference = 0.013

Table 5. Analysis of variance of the standard plate count and Petrifilm SM methods

Source	Sum of sqs	Degrees of freedom	Mean sq.	*F*-ratio
		Standard Plate Count		
Laboratories	0.43197	10	0.04320	16.04[a]
Samples	6.40166	7	0.91452	339.49[a]
Laboratories by samples	0.18857	70	0.00269	1.03
Error	0.22928	88	0.00261	—
Total	7.25147	175	—	—
		Petrifilm SM Method		
Laboratories	0.21114	10	0.02111	5.27[a]
Samples	5.45840	7	0.77977	194.60[a]
Laboratories by samples	0.28049	70	0.00401	2.03[a]
Error	0.17383	88	0.00198	—
Total	6.12386	175	—	—

[a]Significant at the 1% level.

onto center of film base. Carefully roll top film down onto inoculum. Distribute sample over prescribed growth area with downward pressure on center of plastic spreader device (recessed side down). Leave plate undisturbed 1 min to permit gel to solidify. Incubate plates 48 ± 3 h at 32° ± 1°.

In incubator, place plates in horizontal position, clear side up, in stacks not exceeding 10 units. Count plates promptly after incubation period. If impossible to count at once, store plates after required incubation at 0–4.4° for not >24 h. This should be avoided as a routine practice.

Use std colony counter for counting purposes. Magnifier-illuminator may also be used to facilitate counting. Colonies stain in various shades of red. Count all colonies in countable range (30–300 colonies).

To compute bacterial count, multiply total number of colonies per plate (or av. number of colonies per plate if counting duplicate plates of same diln) by reciprocal of diln used. When counting colonies on duplicate plates of consecutive dilns, compute mean number of colonies for each diln before detg av. bacterial count. Estd counts can be made on plates with >300 colonies and should be reported as estd counts. In making such counts, circular growth area can be considered to contain ca twenty 1 cm squares. To isolate colonies for further identification, lift top film and pick colony from gel.

Table 6. Analysis of variance of the VRBA and Petrifilm VRB methods for coliform enumeration

Source	Sum of sqs	Degrees of freedom	Mean sq.	*F*-ratio
		Violet Red Bile Agar		
Laboratories	0.48394	10	0.04839	3.51[a]
Samples	22.84531	7	3.26362	236.61[a]
Laboratories by samples	0.96553	70	0.01379	0.65
Error	1.87736	88	0.02133	—
Total	26.17215	175	—	—
		Petrifilm VRB Method		
Laboratories	0.10981	10	0.01098	0.63
Samples	24.68524	7	3.52646	203.40[a]
Laboratories by samples	1.21363	70	0.01734	0.91
Error	1.67736	88	0.01906	—
Total	27.68605	175	—	—

[a]Significant at the 1% level.

Table 7. Summary of repeatability and reproducibility of the methods evaluated in this study

Statistic	Method			
	SPC	Petrifilm SM	VRBA	Petrifilm VRB
Repeatability variance	0.00261	0.00198	0.02133	0.01906
Reproducibility variance[a]	0.00518	0.00407	0.02349	0.01906
Repeatability standard deviation	0.05104	0.04444	0.14606	0.13806
Reproducibility standard deviation	0.07197	0.06380	0.15326	0.13806
Repeatability coefficient of variation, %	2.17	1.87	11.85	11.32
Reproducibility coefficient of variation, %	3.06	2.68	12.43	11.32

[a]Sum of between-replicates (repeatability) variance, interaction variance, and between-laboratory variance.

(b) *Coliform count.*—Use Petrifilm VRB or equiv. plates. Proceed as in (a), but distribute sample over plate by using plastic spreader, flat side down. Incubate plates 24 ± 2 h at 32° ± 1°. Count as in (a), but count only red colonies that have one or more gas bubbles associated (within 1 colony diam.) with them. Count all colonies in countable range (15–150 colonies). Red colonies without gas bubbles are not counted as coliform organisms.

Results and Discussion

Bacterial counts were first converted to $\log_{10}$ counts/mL. These converted counts were assumed to be normally distributed and of homogeneous variance. Components of variance estimates were calculated using standard statistical techniques, which are described in ref. 4. Repeatability and reproducibility estimates were calculated according to procedures outlined in the *Statistical Manual of the AOAC* (5). Both laboratory and sample effects were assumed to be random. All statistical tests were carried out at the 1% level of significance.

Tables 1 and 2 show the actual counts/mL and Tables 3 and 4 show the mean $\log_{10}$ counts/mL for the 4 methods evaluated in this study. Overall mean $\log_{10}$ counts/mL were 3.377 and 3.350 for the Petrifilm SM and SPC methods, respectively. The difference (0.027) was not statistically significant (1% level). Overall mean $\log_{10}$ counts/mL for the Petrifilm VRB and VRBA methods were 1.220 and 1.233, respectively; the difference (0.013) was not significant.

Results of an analysis of variance of each of the 4 methods are shown in Tables 5 and 6. Significant laboratory effects were observed for Petrifilm SM, SPC, and VRBA methods. The Petrifilm SM also reflected a significant interaction effect.

Based on these analyses of variance, estimates of between-replicates variance, interaction variance, and between-laboratories variance were calculated. Table 7 summarizes these estimates as repeatability variance (between-replicates (intralaboratory) variance) and reproducibility variance (sum of between-replicates variance, interaction variance, and between-laboratories variance) for each of the 4 methods. Corresponding standard deviations and coefficients of variation are also shown. No significant difference was found in either repeatability or reproducibility between the Petrifilm SM and SPC methods, or between the Petrifilm VRB and VRBA methods. Furthermore, both Petrifilm SM and SPC methods show repeatability and reproducibility variances of less than 0.005 and 0.012, respectively, the criteria for acceptability of alternate procedures set forth in *Standard Methods for the Examination of Dairy Products* (1).

Recommendation

The Co-Associate Referees recommend adoption of the Petrifilm SM and Petrifilm VRB (dry rehydratable film) methods for the enumeration of total bacteria and coliform count in milk as official first action.

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DNA Colony Hybridization Method Using Synthetic Oligonucleotides to Detect Enterotoxigenic *Escherichia coli:* Collaborative Study

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The genes that encode several of the enterotoxins produced by *Escherichia coli* have been cloned by recombinant DNA techniques. When the nucleotide sequence of these genes is determined, defined sequence oligonucleotides that include a part of these genes may be synthesized. A 22-base DNA hybridization probe was produced for each of 2 heat-stable *E. coli* enterotoxin (ST) genes: STH, from strains originally isolated from humans; and STP, from strains first found in pigs. For this study, ^{32}P end-labeled DNA probes, sonicated calf thymus DNA, and 3 known and 20 unknown (10 ST-positive and 10 ST-negative) strains were sent to each of 23 collaborators. Cultures were spotted onto an agar-based medium and grown into colonies, which were transferred by blotting to cellulose filters, lysed by alkali and steam, and used for DNA colony hybridization with the ST DNA probes. Strains containing an ST gene were recognized as dark spots on an autoradiogram. Of the 460 samples analyzed, 440 (95.7%) were correctly classified by the collaborators. The method has been adopted official first action.

The feasibility of using DNA colony hybridization to identify *Salmonella* (1) or virulent strains of *Escherichia coli* and *Yersinia enterocolitica* (2, 3) in foods has been established. The method was studied collaboratively (4) and adopted official first action (5). Two disadvantages of the method, however, are the need to isolate and purify cloned gene fragments for specific enterotoxin genes (6) and the use of nitrocellulose filters (7), which are costly and may become brittle and difficult to handle.

Two recent technical advances have helped to overcome both of these difficulties. Defined sequence oligonucleotides can now be synthesized in vitro (8), and cellulose filters have been used successfully for DNA colony hybridization with a simplified protocol (9). This collaborative study tested the reliability of these changes in the hybridization procedure. The filter preparation and DNA hybridization conditions are used in place of those specified in secs **46.044–46.048** (5). If an appropriate synthetic oligodeoxyribonucleotide probe for the heat-labile enterotoxin (LT) of *E. coli* is available, it is recommended that the present method be substituted for secs **46.035–46.048** (5). If other probes or organisms are used, hybridization temperatures (**46.045**) and culture growth conditions (**46.044**) may need to be altered.

Some strains of *E. coli* elaborate protein enterotoxins, which, even after being boiled (10), induce fluid accumulation in suckling mice. At least 2 similar heat-stable enterotoxins (ST) have been identified: STP was first discovered in strains initially isolated from pigs; STH was first characterized in strains obtained from humans. The nucleotide sequences of both these enterotoxins have been determined (11, 12), facilitating the synthesis of oligodeoxyribonucleotide hybridization probes for a portion of the structural gene for each of the toxins. The probes were constructed to be specific for either STH or STP. Although it may be convenient to identify the particular enterotoxin synthesized by a given isolate, it was not necessary, for screening purposes, to determine whether a strain carried the genetic potential to produce STH or STP. Therefore, although 2 probes were used, they were combined into a single pool both for labeling and for hybridization, and were referred to collectively as the ST probe. Preliminary experiments demonstrating the conditions for use and the efficacy of these 2 probes have been described (13).

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This report of the Associate Referee, W. E. Hill, was presented at the 99th AOAC Annual International Meeting, Oct. 27–31, 1985, at 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* (1986) 69, March issue.

Table 1. Bacterial strains used in the study[a]

Culture	Strain	Reference	Enterotoxin[b] STH	Enterotoxin[b] STP
1	BC2	14	+	−
2[c]	H10407	15, 16	+	+
3	B41	17	−	+
4	K334C2	18	−	−
5	TD427C2	19	−	−
6	K324C1	19	−	−
7	35897	19	+	+
8	TD412C1	19	+	−
9	53892A2	19	−	+
10	408-4	19	−	−
11	M415C5	18	−	−
12	M421C5	19	−	−
13	B44	19	−	+
14	E2534	20	−	−
15[c]	HB101(pSLM004)	12	+	−
16[c]	ATCC 25922	21	−	−
17	ATCC 14028 CDC6516-60	—	−	−
18	ATCC 25923	21	−	−
19	B41	17	−	+
20	154165-2	22	+	−

[a]Strains are *Escherichia coli*, except cultures 17 *(Salmonella typhimurium)* and 18 *(Staphylococcus aureus)*.
[b]Determined by hybridization to the cloned STH and STP specific probes.
[c]These cultures were also used as controls.

Collaborative Study

The cultures used in the study are described and their sources (14–22) are listed in Table 1. Each of the 23 collaborators received 3 labeled control cultures (positives, H10407 (15, 16) and HB101(pSLM004) (12); negative, ATCC 25922); and 20 coded unknown cultures (10 positive, 10 negative). Cultures were coded by computer-generated random numbers that were uniquely assigned to each laboratory. Each collaborator also received about 5×10^6 cpm ^{32}P-labeled synthetic oligodeoxyribonucleotide ST gene probes (equal amounts of STH and STP), and 1 mL sonicated calf thymus DNA. Collaborators conducted the culture handling and hybridization filter preparation steps as well as the colony hybridization and all subsequent procedures.

If the intensity of spots on autoradiograms was greater than that of the negative control and approximately equal to or greater than that of the positive controls, that culture was considered to be positive for the presence of at least one of the ST genes. Collaborators submitted autoradiograms to the Associate Referee along with culture results.

Enterotoxigenic *Escherichia coli*

DNA Colony Hybridization Method Using Synthetic Oligodeoxyribonucleotides and Paper Filters

First Action

(*Caution:* This procedure uses radioactive compd. Personnel must receive adequate training and monitoring and have proper facilities available for handling this substance.)

46.B08 ***Principle***

Chemically synthesized pieces of DNA (oligodeoxyribonucleotides) that code for regions of genes detg bacterial virulence can be used to identify pathogenic strains of bacteria. These oligomers are radioactively labeled in vitro and hybridized with colonies of bacterial cells that have been lysed and fixed to paper filters. Colonies contg same region of a gene will bind labeled DNA and become radioactive. Such colonies can be detected by autoradiography.

46.B09 ***Reagents***

(Prep. all media according to manufacturer's instructions and use analytical grade materials whenever possible. Note: DNA often adheres to unsiliconized glass. When working with solns contg DNA, use siliconized glassware or disposable plasticware unless otherwise specified.)

(a) *Lysis mixture A.*—Combine 50 mL 10N NaOH, (**s**), 300 mL 5.0M NaCl, (**u**), and 650 mL H_2O.

(b) *Lysis mixture B.*—Combine 50 mL 2.0M Tris, pH 7.0, (**v**), 400 mL 5.0M NaCl, (**u**), and 550 mL H_2O.

(c) *Hybridization mixture.*—Combine in plastic tube or beaker: 28.9 mL H_2O, 15.0 mL 20X SSC, (**d**), 5.0 mL 50X Denhardt's soln, (**e**), and 0.1 mL 0.5M EDTA soln, pH 8.0, (**f**). Final vol. is 49 mL. Use immediately.

(d) *20X std saline citrate soln (SSC).*—Dissolve 175.4 g NaCl and 88.2 g Na citrate in final vol. of 1 L H_2O.

(e) *50X Denhardt's soln.*—Dissolve 2.0 g Ficoll (av. molecular wt 400 000), 2.0 g polyvinyl pyrrolidone (av. molecular wt 360 000), and 2.0 g bovine serum albumin in 200 mL H_2O. Store at −20° in 5.0 mL aliquots.

(f) *0.5M Disodium ethylenediamine tetraacetate soln, pH 8.0.*—Dissolve 186.12 g Na_2EDTA in 800–900 mL H_2O. Adjust to pH 8.0 with 10N NaOH, (**s**). Dil. to 1 L with H_2O.

(g) *Sonicated calf thymus DNA.*—Dissolve 1 g purified calf thymus DNA in 100 mL H_2O by stirring 3–4 h. Sonicate until av. molecular wt is 300 000–500 000, which may be detd by electrophoresis with appropriate stds such as 123-base ladder (Bethesda Research Laboratories (BRL), Gaithersburg, MD). Store in 1 mL portions in 13 × 100 mm screw-cap tubes. Glass may be used in this instance only.

(h) *6X SSC soln.*—Combine 300 mL 20X SSC, (d), with 700 mL H_2O.

(i) *2X SSC soln.*—Combine 100 mL 20X SSC, (**d**), with 900 mL H_2O.

(j) *Synthetic DNA stock soln.*—Approx. 150–350 μg/mL. (A_{260} = 5–10 units.) Soln of 22-base, single stranded DNA molecules [STH (human) and STP (porcine) oligodeoxyribonucleotide probes for enterotoxin genes] will have concn ca 20–50μM. Store at −20°.

(k) *Synthetic DNA working soln.*—Dil. stock soln, (**j**), in H_2O to 10μM. Store at −20°.

(l) *2.0M Tris soln, pH 7.6.*—Dissolve 242.28 g Tris in ca 800 mL H_2O. Adjust to pH 7.6 with concd HCl. Dil. to 1 L with H_2O.

(m) *1.0M $MgCl_2$ soln.*—Dissolve 9.52 g $MgCl_2$ in final vol. of 100 mL H_2O.

(n) *0.5M Dithiothreitol soln.*—Weigh 0.77 g dithiothreitol and combine with H_2O to final vol. of 10.0 mL. Store at 4°.

(o) *10mM Spermidine soln.*—Dissolve 14.5 mg spermidine in final vol. of 10.0 mL H_2O. Store at −20°.

(p) *10X Kinase buffer.*—Combine 2.5 mL 2.0M Tris, pH 7.6, (**l**), 1.0 mL 1.0M $MgCl_2$, (**m**), 1.0 mL 0.5M dithiothreitol, (**n**), 1.0 mL 10mM spermidine, (**o**), 20 μL 0.5M EDTA, (**f**), and 4.5 mL H_2O. Store at 4°.

(q) *(γ-^{32}P) ATP.*—Aq. soln of adenosine triphosphate, specific activity 3000–7000 Ci/mmole. ("Crude" prepn from ICN Pharmaceuticals, Inc., Irvine, CA 92713, or equiv.). Store at −70° if possible.

(r) *Bacteriophage T4 polynucleotide kinase.*—20 units/μL (BRL or equiv.).

(s) *10N NaOH soln.*—Dissolve 400 g NaOH in final vol. of 1 L H_2O.

(t) *2.0M Tris soln, pH 8.0.*—Follow instructions for (**l**) but adjust pH to 8.0.

(u) *5.0M NaCl soln.*—Dissolve 292.2 g NaCl in final vol. of 1 L H_2O.

(v) *2.0M Tris soln, pH 7.0.*—Follow instructions for (**l**) but adjust pH to 7.0.

(w) *Glycerol freezing soln.*—Combine 50.0 mL glycerol and 50.0 mL H_2O. Dispense 0.5 mL aliquots into 1 dram vials. Sterilize by autoclaving 15 min at 121°.

(x) *NACS PREPAC column loading buffer.*—Dissolve 308.4 g ammonium acetate in final vol. of 1 L H_2O.

(y) *NACS PREPAC column eluting buffer.*—Dissolve 19.3 g ammonium acetate in final vol. of 1 L H_2O.

(z) *Brain heart infusion or trypticase soy broth and agar.*—For microbial growth.

(aa) *Scintillation fluid.*—Dissolve 5.0 g 2,5-diphenyloxazole in 1 L toluene.

(bb) *ST probe soln.*—Combine equal vols of STH and STP working soln, (k).

(cc) *Phosphoramidite soln.*—0.5 g (Applied Biosystems, Inc., Foster City, CA 94404; American BioNuclear, Emeryville, CA 94608; or equiv.), reagent grade (≥95%), made up to 0.1M using anhyd. CH_3CN, (nn), and glass syringe transfer procedures with protection from atm. H_2O. Vortex mix until dissolved.

(dd) *Thiophenol soln.*—Mix 80 mL *p*-dioxane (≤0.01% H_2O), 80 mL triethylamine (99+%), and 40 mL thiophenol (99+%) ("Gold Label," Aldrich Chemical Co., or equiv.).

(ee) *1H-Tetrazole soln.*—Add 300 mL anhyd. CH_3CN, (nn), to 10 g resublimed tetrazole, (oo), with protection from atm. H_2O, and sonicate until dissolved. Warm (30–40°), if necessary.

(ff) *Ammonium hydroxide soln.*—28–30% NH_3, as supplied.

(gg) *Acetic anhydride soln.*—Combine 160 mL tetrahydrofuran (≤0.01% H_2O), 20 mL 2,6-lutidine ("Spectro Grade," Eastman Kodak Co., or equiv), and 20 mL acetic anhydride (99+%).

(hh) *4-Dimethylaminopyridine soln.*—Dissolve 13 g recrystd 4-dimethylaminopyridine, (pp), in 200 mL tetrahydrofuran (≤0.01% H_2O).

(ii) *Trichloroacetic acid soln.*—Weigh 125 g trichloroacetic acid (Aldrich "Gold Label" or equiv., 99+%) in beaker with min. exposure to atm. moisture and transfer to storage container using 4 L CH_2Cl_2 (≤0.006% H_2O).

(jj) *Iodine soln.*—Combine 320 mL tetrahydrofuran, 80 mL 2,6-lutidine, and 10.2 g I crystals. Sonicate until dissolved. Add 8.0 mL H_2O, dropwise, with stirring.

(kk) *Dimethoxytrityl (DMT) assay soln.*—Dissolve 19 g *p*-toluenesulfonic acid monohydrate in 1 L LC grade CH_3CN (0.1M).

(ll) *Triethylammonium acetate (TEAA) buffer*—With const stirring, add 28 mL triethylamine, (qq), to 1.8 L H_2O followed by 10 mL glacial acetic acid. Titr. slowly with more acid to pH 7.0 and then vac. filter thru type HA 0.45 μm filter (Millipore Corp. or equiv.).

(mm) *Detritylation soln.*—Add 3 mL glacial acetic acid to 97 mL H_2O.

(nn) *Anhydrous acetonitrile.*—Store 1 L LC grade CH_3CN (≤0.007% H_2O, Burdick & Jackson Laboratories, Inc., or equiv.) over type 4A molecular sieves ≥24 h.

(oo) *Resublimed 1H-tetrazole.*—Sublime 20 g 1*H*-tetrazole (99+%, Aldrich "Gold Label" or equiv.) in std sublimation app. at ≤0.25 torr and 130–140°. (Yields ca 15 g sublimate.)

(pp) *Recrystallized 4-dimethylaminopyridine.*—Dissolve 200 g 4-dimethylaminopyridine in ca 1 L hot (50–60°) tetrahydrofuran contg 20 g decolorizing charcoal. Filter while still hot thru glass fiber paper (Grade 934AH, "Reeve Angel," Whatman, Inc., or equiv).

(qq) *Triethylamine*—99+% (Aldrich "Gold Label" or equiv. LC grade).

46.B10 *Apparatus and Materials*

(a) *Labware.*—100 × 15 mm glass petri plates; plastic beakers and tubes to contain up to 100 mL; 100 × 15 or 20 mm plastic petri plates; plastic conical tubes to contain up to 500 μL; plastic pipets to cover range 1–10 mL; variable vol. micropipettors and tips to cover range 1–1000 μL.

(b) *Incubators.*—(*1*) Capable of maintaining 37 ± 1°; (*2*) capable of maintaining 40 ± 1°; (*3*) capable of maintaining 50 ± 1°; (*4*) H_2O bath or dry block capable of maintaining 37 ± 1°.

(c) *UV spectrophotometer.*—To measure DNA concn at 260 nm. (1 A_{260} unit is 50 μg/mL for double stranded DNA and 33 μg/mL for single stranded DNA.)

(d) *Ultralow temperature freezer.*—Capable of maintaining −70° is preferred, but freezer (not frost-free) at −20° may be substituted.

(e) *Freezer.*—Capable of maintaining −20° (not frost-free).

(f) *Cellulose filters.*—No. 541 (Whatman), 82–85 mm diam.

(g) *Absorbent filters.*—Whatman No. 1 or similar, ca 85 mm diam.

(h) *NACS PREPAC column.*—DNA binding resin (BRL or equiv.).

(i) *Scintillation counter.*—Or Geiger-Mueller counter if calibrated in cpm.

(j) *X-ray film and developing chemicals.*—8 × 10 in. is convenient size. Kodak XAR X-ray film or equiv.

(k) *Darkroom.*—Facilities for X-ray film development with appropriate safelight.

(l) *X-ray film holder cassette.*—With intensifying screens (Kodak regular, Eastman Kodak Co.; Dupont Cronex Lightening Plus, E.I. Dupont de Nemours & Co.; or equiv.).

(m) *Centrifuge.*—Capable of spinning 500 μL conical plastic tubes (Eppendorf Model 5412, Brinkmann Instruments, Inc., or equiv.).

(n) *Vacuum desiccator.*—Needed only if prepd colony hybridization filters must be stored 1 week.

(o) *DNA synthesizer.*—Manual or automated synthesis system (i.e., Applied Biosystems synthesizer Model 380A; other synthesis systems providing equiv. results are also acceptable).

(p) *Synthesis ("reaction") columns.*—1 μmol long chain alkylamine-functionalized controlled pore glass, either prepacked or hand-packed (Applied Biosystems or equiv.).

(q) *Fraction collector.*—To collect fractions from automated synthesis system. Should have auxilliary signal input.

(r) *Liquid chromatographic system.*—App. with gradient elution capability, UV detection at 254 or 260 nm, and μBondapak® C_{18}, 7.8 mm × 30 cm column (Waters Associates, Inc., or equiv.).

(s) *Rotary vacuum centrifuge.*—To conc. LC-purified oligodeoxyribonucleotides (Savant, Hicksville, NY 11801, or equiv.).

(t) *Glass syringes.*—Capacity up to 10 mL for transfer of anhyd. CH_3CN with protection from atm. moisture.

(u) *Type HV, 0.45 μm filters.*—To remove LC column particulates (Millipore or equiv.).

46.B11 *Colony Hybridization Filter Preparation*

Transfer candidate cultures to 5 mL brain heart infusion or trypticase soy broth and incubate 18–24 h at 37°. If culture must be stored before analysis can be performed, aseptically add 2.0 mL culture to 0.5 mL freezing soln, (w). Store at −70° if possible. (Note: Frost-free freezers will decrease culture viability and may result in loss of virulence determinants. If cultures must be stored at −20°, use non-frost-free unit. This precaution holds for all frozen material in this procedure.)

Aseptically inoculate 5 mL rich broth with portion of frozen bacterial culture. Sterile cotton swabs are well suited for this purpose. Always include known pos. and neg. control cultures on every filter (see below). (If culture is not thawed, it may be reused innumerable times.) Incubate culture 18–24 h at 37°. At same time, aseptically prepare 100 × 15 mm petri plates contg either brain heart infusion or trypticase soy agar and dry inverted 18–24 h at 37°. After inoculating cultures in orderly array and ensuring that resulting colonies will not ultimately merge while growing, inoculate agar plates with test cultures, using sterile microbiological needle, toothpick, cotton swab, or replicator; 9–10 mm is convenient distance between cultures. Record location of each culture; it is vital that culture patterns and resulting autoradiogram(s) can be oriented unambiguously. Prep. multiple plates and concomitant filters because hybridization procedure may have to be repeated and number of steps to be repeated is thereby lessened. Incubate plates inverted 18–24 h at 37°. Mark cultures failing to grow; otherwise, false-neg. results may be reported.

Label Whatman No. 541 cellulose filters, (f), 82–85 mm diam., using soft lead pencil, and also mark filter so it can be oriented unambiguously after replication. (Note: Other manufacturers make filters with physical properties equiv. to Whatman No. 541. However, DNA binding abilities of such filters are not always suitable for use in DNA hybridization.) Apply filter so that side with pencil markings faces colony array on agar surface of plate contg colonies. Wetting initial edge of filter paper and rolling to opposite edge usually eliminates formation of air pockets. If air bubbles are entrapped between filter and agar plate, remove by applying gentle

pressure with glass spreader. This maneuver also ensures more efficient attachment of cultures to filter paper, but care must be taken to avoid spreading colonies because of excessive pressure. Filters may be peeled from plate immediately, but more definitive reactions are usually obtained if filter remains situated 1–2 h. (Note: Colony array on filter is now mirror image of array originally applied to agar plate.)

Lyse colonies replicated onto filters by transferring filters with colony side up onto absorbent cellulose filters (such as Whatman No. 1 or S & S No. 597, ca 85 mm diam.) contained in glass 100 × 15 mm petri plates and previously wetted with 1.5–2.0 mL lysis mixt. A, (**a**). Be sure that no air is entrapped between filters. Heat filters in glass plate for 3–5 min in steam. Transfer steamed filters to glass petri plates contg absorbent cellulose filters previously wetted with 1.5–2.0 mL lysis mixt. B (**b**). Again, be sure that no air pockets result. Maintain filters in horizontal position when transferring so that lysed colonies (DNA) will not become confluent. Let filters become completely neutralized by remaining situated 5–10 min.

If filters are not to be used immediately, air-dry on absorbent paper at room temp. and store under vac. between filter papers. Such filters have been kept ca 1 year without noticeable change in results.

46.B12 *Oligodeoxyribonucleotide Synthesis*

(Note 1: A number of companies will custom-synthesize oligodeoxyribonucleotides. Also, several oligodeoxyribonucleotide synthesis systems are com. available, both automated and manual. Results are generally satisfactory if manufacturer's instructions are followed. This method uses one of com. available, automated synthesizers and procedure described below is meant to serve only as example.)

(Note 2: All solns for prepn and isolation of synthetic oligodeoxyribonucleotides should be prepd in deionized H_2O passed thru 0.2 μm filter ("Versacap Filter Unit," Gelman Sciences, Ann Arbor, MI 48106, or equiv.).)

According to manufacturer's instructions, use Applied Biosystems, "fast" cycle but with following modifications of step times: trichloroacetic acid to column detritylation step, 75 s (retained in fraction collector); CH_3CN to column post-detritylation step, 50 s (also retained and pooled with above in fraction collector); CH_3CN to column, pre-coupling step, 120 s; coupling step, 180 s; capping step, 120 s. Synthesis is ended with dimethoxytrityl (DMT) group retained at 5′ terminus. Automated cleavage from support is achieved with concd NH_4OH at room temp. for 1 h. Dil. delivered NH_4OH soln with 1 mL concd NH_4OH, heat 10 h at 60° in 3.7 mL vial with Teflon-lined screw cap (Supelco, or equiv.). Let cool to room temp. Add 50 μL triethylamine (**qq**). Evap. NH_3 with N stream to ca 2 mL.

46.B13 *Quantitation of Coupling Yield*

To det. isolated product yield (see below) and ensure satisfactory coupling at each addn, theoretical yields of product must be calcd. Dil. each collected fraction (from detritylation and post-detritylation steps above) to 5 mL with DMT-assay soln (**kk**). Mix each fraction well and read *A* at 530 nm. Use assay soln (**kk**) as reference std. Compare *A* with that of previous fraction to det. coupling efficiency of each step (generally 97–99%). To det. overall theoretical yield, multiply all individual step-yields.

46.B14 *Oligodeoxyribonucleotide Purification and Isolation*

To det. chromatgc properties of prepn, perform anal. run. Set detector for 0.1 AUFS. Inject 10 μL soln evapd to 2.0 mL. In ambient temp. column, start 20–30% gradient (at 1%/min) of CH_3CN in triethylammonium acetate buffer, (**ll**). Continue at 30% CH_3CN after 10 min. Generally, major DMT-product elutes at 10 ± 3 min. After elution time is detd, repeat chromatgy on preparative scale (inject 100 μL crude soln, 1.0 AUFS). Collect center position of major peak.

46.B15 *Oligodeoxyribonucleotide Processing*

Before synthetic oligonucleotide can be used as substrate for polynucleotide kinase, LC solvs and DMT group must be removed. Conc. collected LC fraction using N ca 10–20 min to remove most CH_3CN. Conc. sample to dryness using rotary vac. centrf., (s). Add 1 mL 3% (v/v) acetic acid to remove DMT protecting group. Vortex-mix to dissolve. After 5–10 min at room temp., freeze in crushed dry-ice and conc. using vac. centrf., (s). Dissolve residue in 1 mL H_2O. Add 1 mL anal. grade ethyl acetate to ext org. impurities and vortex-mix thoroly. Let org. layer sep. from aq. layer contg DNA and possible LC column particulates. (Centrf. if necessary.) Remove org. layer with Pasteur pipet and discard. If insoluble LC column particulates are present, syringe-filter DNA soln thru type HV, 0.45 μm filter (**u**). Let DNA soln gravity-filter and collect residual soln by rapidly depressing syringe plunger. Remove 50 μL aliquot from 1 mL filtered DNA soln for *A* measurement. Conc. both remaining sample and *A* aliquot to dryness. Dissolve aliquot in 1 mL H_2O and measure *A* at 260 nm. Since 1/20 of sample has been removed, multiply reading by 19 to obtain *A* units in total purified sample. Discard *A* aliquot. Multiply *A* in total purified sample by 10 (because only 10% of total synthesis reaction was purified) to obtain *A* units of entire isolable product. Compare this yield with calcd value (1 μmole × theoretical yield [see above] × molar *A* of oligonucleotide synthesized × 10^{-3}) to det. yield of isolable product. Molar *A* is calcd by adding number of purines (dA plus dG) times 14 000 plus number of pyrimidines (dC plus T) times 7000. These factors are molar extinction coefficients and 10^{-3} is used to convert molar *A* to μmoles/mL which is a millimolar concn.

46.B16 *End-Labeling of Synthetic DNA*

Synthetic oligodeoxyribonucleotides are rehydrated to ca 5–10 A_{260} units (ca 150–350 μg/mL) to serve as stock soln (**j**). One A_{260} unit corresponds to ca 33 μg/mL single-stranded DNA. Molecular wt of 22-base, single-stranded DNA molecule is ca 7260. Prep. 10 μM working soln for each DNA probe (10 pmoles/μL, 72.6 μg/μL). If desired, STH and STP synthetic DNA probes can be combined into single soln, 5μM in each probe (**bb**).

Mix 5 μL DNA probe soln, (**bb**), 2.5 μL 10X kinase buffer, (**p**), 15 μL H_2O, 1.5 μL (γ-^{32}P) ATP, (**q**), and 1 μL T4 kinase, (r), in 500 μL plastic conical centrf. tube, (**a**), on ice. Add kinase, (r), last and return enzyme immediately to −20° because it is quite heat-labile. Centrf., (**m**), 2–3 s to adequately mix reagents. Incubate at 37° in H_2O bath or dry block heater, (**b**), 1 h. Add 2 μL 0.5M EDTA, (**f**), to terminate reaction. Add 1.6 μL 4.0M ammonium acetate soln, (**y**), to bring ammonium acetate concn to 0.25M before applying sample to NACS PREPAC column.

Unincorporated ^{32}P is removed by binding DNA to NACS PREPAC column, (**h**). Equilibrate column with 0.25M ammonium acetate, (**x**), 2 h. Load reaction mixt. onto column and wash, using gravity or very gentle pressure, with ca 4 mL loading buffer, (**x**), to remove free ATP. Elute bound DNA with 200 μL aliquots of eluting buffer, (**y**). Do not force liq. thru column rapidly. Collect three 200 μL fractions in 500 μL plastic tubes, (a). Spot 2 μL of each fraction onto ca 2 × 2 cm paper (e.g., Whatman 3MM), dry, add ca 5 mL scintillation fluid, (**aa**), and assay radioactivity by scintillation counting. Geiger-Mueller counter, (**i**), may suffice if properly calibrated and used. Most labeled DNA is eluted from column in fractions 1 and 2. Pool fractions and count triplicate 2 μL portions as described above. Est. total vol. of prepn by carefully drawing into plastic 1 mL pipet. Calc. total amt of radioactivity recovered in prepn. Usually, 1–2 × 10^8 cpm is obtained if specific activity of ATP, (**q**), is 3000–7000 Ci/mmol. Store at −20°.

46.B17 *Colony Hybridization*

Freshly prep. 50 mL hybridization mixt., (**c**). Boil 1.0 mL sonicated calf thymus DNA, (**g**), 5 min in H_2O bath and add to hybridization mixt., (**c**). Dispense 10 mL sonicated calf thymus DNA-hybridization mixt. into 100 × 15 or 20 mm *plastic* petri dish and insert cellulose filter contg lysed colony array. To use std amt of probe for each hybridization, det. vol. of probe DNA soln required to contain 1 ×

10^6 cpm after correcting for 14.2 day half-life of ^{32}P. Add 1×10^6 cpm probe DNA to soln contg filter. Mix briefly and incubate plate overnight at 40°.

Wash hybridized filters free of ^{32}P-labeled DNA not specifically bound to DNA from colonies on filter by removing filter from hybridization mixt. and rinsing 5–10 s in plastic petri dish contg 10 mL 6X SSC, (h). Drain and recover filter with 6X SSC. Incubate 1 h at 50°. Again, drain plate, recover with 6X SSC, and incubate 1 h at 50°. Finally, rinse filter 5–10 s at room temp. in 2X SSC, (i). Air-dry on absorbent paper at room temp. to prevent curling. Mount filter to 8 × 10 in. stiff paper (e.g., Whatman 3MM) using small pieces of tape. Cover with plastic or glassine sheet (such as document or neg. holder) to prevent contamination of intensifying screens in X-ray film holders.

46.B18 *Autoradiography*

Exposure time is dictated by amt of radioactive DNA bound to filter. If increase above background exceeds 10 cps when Geiger-Mueller counter is held over filter, it is likely that pos. reaction will be visible after 4 h exposure at room temp. However, if increase of 2–3 cps is observed, enclose loaded film cassette in sealed plastic bag and expose film overnight, preferrably at −70° or at least −20°. If −70° is not available, cassette can be sandwiched between slabs of dry ice to reduce exposure time.

In darkroom, place X-ray film onto plastic-covered filter in cassette film holder with intensifying screens. Expose film for appropriate length of time as detd above. After exposure, let cassette equilibrate at room temp. (to prevent moisture accumulation) before removing plastic bag. Develop X-ray film by following manufacturer's instructions. If spots are too faint or too intense for analysis, expose new film for appropriate length of time.

46.B19 *Reporting of Results*

Lysed colonies of *E. coli* strains contg DNA coding for heat-stable enterotoxins will bind radioactively labeled oligonucleotide probe for ST. These radioactive lysed colonies will expose X-ray film and dark spots will be evident after development. Det. if each unknown culture is pos. or neg. by comparing spot intensity to that of pos. and neg. culture controls. However, many factors can influence quality of these results: size of colonies, amt of cellular debris, amt of DNA per lysed colony, hybridization and washing temps, hybridization time, specific activity of probe, and length of autoradiogram exposure. Well documented pos. and neg. controls must be present on every filter to ensure that the procedure has been performed correctly and that compensation for nonspecific binding of labeled probe DNA (neg. colonies that may be seen as faint spots) has been made.

If neg. control cultures exhibit faint spots, and pos. culture spots are intense, re-wash filter(s) in 6X-SSC, (h), at 52–55° twice for 1 h each time. Dry filters and re-expose autoradiogram. Take care because thermal stability of oligonucleotide hybrids is much less than that of longer DNA molecules.

46.B20 *Troubleshooting*

Unsatisfactory autoradiograms can result from several factors, some of which have been listed in the previous section. False-neg. results can be due to spontaneous loss of plasmids, especially when strains are cultivated excessively under nonselective laboratory conditions (i.e., re-isolation or further subculture). Also, hybridization and/or washes at excessively high temps can result in decreased DNA probe binding which in turn can lead to neg. observation. Occasionally, very large colonies do not become affixed to filters and cellular material is lost from hybridization filters. False-pos. results can be observed if either hybridization or washing temp. is too low. Nonspecific DNA probe binding will occur. Autoradiogram exposures of excessive time can result in overemphasis of limited, nonspecific binding of probe to neg. cultures; this may be falsely reported as pos. results. Other possible sources of error and their remedies have been discussed (**46.048**; JAOAC 67, 801(1984)).

Finally, it is essential to note that resulting autoradiogram spot arrays are mirror images of plate inoculation patterns. This is not the case with **46.035–46.048**. Results are accurately read if autoradiograms are reversed (left to right) before interpretation. Films must be marked so that they can be unambiguously oriented with recorded location of each test culture.

Results and Discussion

Of the 23 collaborators participating, 17 (74%) correctly classified all 20 unknown cultures; the accuracy of 20 laboratories (87%) was ≥95%. One laboratory each made 8, 5, and 4 misclassifications. Collaborator misidentifications were as follows: Laboratory B, false negative—culture 1; Laboratory E, false negative—cultures 8 and 19, false positive—cultures 10 and 14; Laboratory H, false positive—culture 18; Laboratory R, false negative—cultures 1, 3, 8, 9, and 15; Laboratory U, false negative—culture 19; Laboratory W, false negative—cultures 3, 8, 9, and 13, false positive—cultures 5, 11, 14, and 17.

Of the 460 test results considered for analysis, 20 cultures were misclassified; 13 of 230 (5.7%) were false negatives and 7 of 230 (3.0%) were false positives. Of the 230 positive culture tests, 217 (94.3%) were correct; 223 (97.0%) of the 230 negative culture tests were correct. There is no statistically significant difference (at the 95% confidence level) in the proportion of incorrect results or the proportions of false negatives and false positives between this study and the previously approved colony hybridization method (4).

From analysis of the autoradiograms and discussions with the collaborators after results were reported, it appears that false-positive results were most frequently due to overexposure of autoradiograms. False-negative results were often reported if cultures had been unnecessarily streaked and cloned onto agar plates in arrays for filter replication.

Conclusions

The advantages and disadvantages of genetic techniques based on DNA colony hybridization for the identification of virulent bacteria have been discussed (23). The study reported here was conducted with pure cultures; however, DNA colony hybridization has also been shown to be applicable directly to several types of food samples (2, 3, 24, 25). The current study demonstrates the reliability of 2 significant improvements made to the previously adopted official method (4, 5): cellulose filters and synthetic oligodeoxyribonucleotide hybridization probes. The difference in accuracy between the 2 methods (96.7 vs 95.7%) is not significantly different.

DNA colony hybridization can be used to identify a large number of bacterial species and strains. Synthetic DNA probes for a number of genes are already in use or being developed. The number will continue to increase as more nucleotide sequences become available for genes that are critical for bacterial identification or that play important roles in microbial pathogenicity.

Recommendation

As a result of this collaborative study (95.7% correct classification of unknown cultures), the Associate Referee recommends that this method be adopted official first action for the identification of strains of *E. coli* harboring the genes encoding STH and/or STP.

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FOOD ADDITIVES

Gas Chromatographic Profile Analysis of Basic Nitrogen-Containing Aromatic Compounds (Azaarenes) in High Protein Foods

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A method is described for the determination of basic nitrogen-containing polycyclic aromatic compounds (N-PACs, azaarenes) in meat. The enrichment procedure includes liquid-liquid partition (dimethylformamide–water–cyclohexane), extraction of N-PACs by sulfuric acid, reextraction after neutralization by cyclohexane or, alternatively, by nonadsorbing ion exchange chromatography. Further purification is performed by column chromatography on Sephadex LH20 using a closed system to avoid sample contamination by laboratory pollutants. N-PACs are analyzed by capillary gas chromatography and measured by comparing to the corresponding peak areas of an internal standard (e.g., 10-azabenzo(a)pyrene). The limit of detection of this method ranges from 0.1 to 0.4 ng for benzacridines, dibenzacridines, and their methyl derivatives. The results of a collaborative study, stimulated by IUPAC, are reported: Coefficients of variation for the various azaarenes were 4.0–13.6% for the check analysis and 10.4–25.4% for a spiked ham sample. Consequently, IUPAC suggests this procedure as a recommended method.

Methods for the determination of basic nitrogen-containing polycyclic aromatic compounds (N-PACs, azaarenes) were developed in the past for some matrixes such as tobacco smoke (1) or petroleum and petroleum products (2) because a number of these compounds, predominantly derivatives of benz(a)– and benz(c)acridine, are well known carcinogens (3–6).

Similar to polycyclic aromatic hydrocarbons (PAHs), basic N-PACs are formed during pyrolysis or incomplete combustion as well as during coalification of nitrogen-containing materials. Consequently, they are found in urban suspended particulate matter (7–10) and in petroleum (for review, see ref. 11). A list of azaarenes isolated from coal tar has been presented by Lang and Eigen (12). On the other hand, the occurrence of azaarenes in tobacco smoke (1) and of pyridine, quinoline, and isoquinoline derivatives in marijuana smoke (13) has been reported. Furthermore, evidence for the formation of mutagenic basic aromatic compounds from aromatic amino acids during broiling of high protein foods has been given (for review, see ref. 14).

Hence, the question arises whether azaarenes also occur in smoked foods or in foliage plants exposed to air pollution. Apart from the information on the occurrence of known compounds, the objective of a gas chromatographic profile method is to spur interest in both characterizing unknown compounds and proving their toxicological significance in foods. To this end, the Food Section of the International Union of Pure and Applied Chemistry (IUPAC) recommended developing a method for the determination of azaarenes, followed by a collaborative study.

Experimental

Note: N-PACs are degraded by UV light; exposure of extracts or standard solutions to sunlight should be avoided.

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Apparatus

Usual laboratory equipment plus the following items: mechanical blender (mincing machine); 30 mm id columns containing 10 g Sephadex LH 20 or 4 g SP-Sephadex C25 (closed system, see Figure 1); concentration tubes (see Figure 2); Perkin-Elmer & Co. gas chromatograph, Model Sigma 2B, or equivalent instrument with flame ionization and/or N-specific detectors.

Gas chromatographic conditions: glass or fused silica capillary column, 0.25 mm × 25 m, coated with polydimethylsiloxane (e.g., silicone SE-30) or 95% polymethyl/5% phenylsiloxane (e.g., J&W DB5). Column should have 50 000 theoretical plates (HETP). Carrier gas, 0.6–0.8 mL helium or nitrogen/min. Flame ionization detector 290°C; injection port 280°C (all glass with a fitting glass tube); column 110–260°C; injection volume 7 μL toluene–pyridine (99 + 1).

Reagents

(a) *Solvents.*—*N,N*-Dimethylformamide (DMF), 1,1,2-trichlorotrifluorethane (TCFE), cyclohexane, isopropanol, methanol distilled in glass (to avoid air contamination, a closed distillation system was used).

(b) *Sulfuric acid.*—55% w/w (pre-extract twice with cyclohexane).

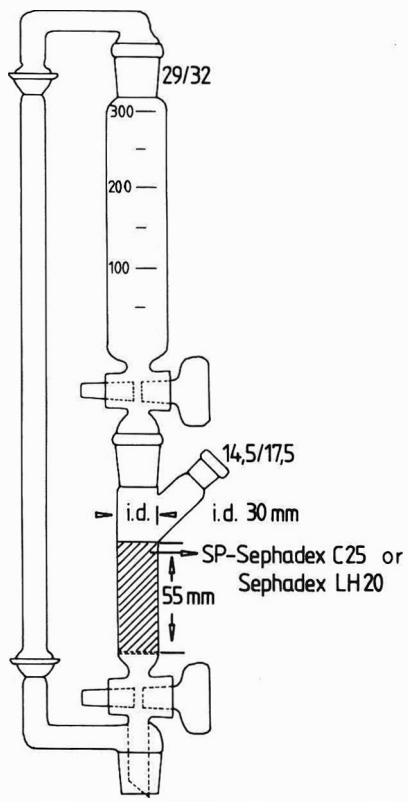

Figure 1. Column for chromatography on Sephadex LH 20 or SP-Sephadex C25 as closed system.

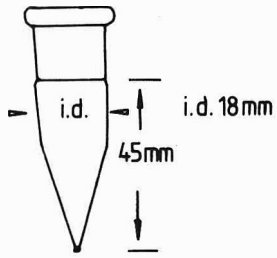

Figure 2. Concentration tube.

(c) *Sodium hydroxide.*—5N (pre-extract twice with cyclohexane).

(d) *Reference N-PACs.*—Benz(c)acridine, dibenz(a,h)acridine, dibenz(a,j)acridine, 10-azabenzo(a)pyrene, all available from Community Bureau of Reference, rue de Loi 200, B-1049 Brussels, Belgium. 8,10-Dimethylbenz(a)acridine (Koch-Light Laboratory). Standard solution: 10-azabenzo(a)-pyrene, 0.1 mg/L cyclohexane.

(e) *SP-Sephadex C25 ion exchanger.*—Activate 4 g with aqueous 200 mL 0.05N hydrochloric acid, wash with 250 mL methanol–water (7 + 3), and then wash with 250 mL methanol. Shake gel bed free of bubbles and compact by soft tapping.

(f) *Sephadex LH 20.*—Equilibrate 10 g with 50 mL isopropanol at least 3 h; add slurry to glass column and let isopropanol drain until liquid reaches top of adsorbent.

Check Analysis

To check purity of all analytical materials used (solvents, adsorbents, etc.), conduct blank test following described procedure, including adding internal standard and reference substances, but excluding sample.

For exhaustive extraction without saponification, sample must be homogenized by mincing machine to homogeneous mash.

Extraction and Liquid-Liquid Partition with DMF/Cyclohexane

Weigh 40 g minced sample in 250 mL round-bottom flask, add 150 mL TCFE and 2.0 mL internal standard solution. Insert condenser, place on heating mantle, and reflux 1 h. Decant solution from slurry and evaporate in rotary evaporator under reduced pressure to ca 5 mL (40°C water bath). Add ca 5 mL cyclohexane and evaporate solvent to 1 mL; then add 39 mL cyclohexane. Transfer this solution to 100 mL separatory funnel and extract with 40 mL DMF–water (9 + 1), shaking ca 5 min; let separate. Transfer lower layer to second 500 mL separatory funnel, add 80 mL water and 120 mL cyclohexane, and shake 5 min. After separation of phases, transfer lower layer to third 500 mL separatory funnel and repeat extraction with 120 mL cyclohexane. Wash collected cyclohexane solutions twice with two 50 mL portions of water. Transfer cyclohexane solution to round-bottom flask and evaporate in rotary evaporator under reduced pressure to 1–3 mL.

Liquid-Liquid Partition with Cyclohexane/H_2SO_4, NaOH/Cyclohexane

Transfer 1–3 mL solution to 20 mL separatory funnel, rinse flask with 2–4 mL portions of cyclohexane, and transfer rinses to funnel. Total volume of cyclohexane should be ca 5 mL. Add 5 mL 55% sulfuric acid and shake mixture 5 min. Let separate and transfer sulfuric acid (lower) layer to 500 mL round-bottom flask. Repeat extraction of cyclohexane solution with 2 mL 55% sulfuric acid, let layers separate, and transfer lower layer to same round-bottom flask. Add 70 mL water dropwise. Add 25 mL 5N NaOH dropwise and cool solution to 5°C, shaking vigorously ca 5 min. Transfer alkaline solution to 250 mL separatory funnel, add 100 mL TCFE, shake 5 min, and let layers separate, Transfer lower (TCFE) layer to another 250 mL separatory funnel, add another 100 mL TCFE to alkaline solution, shake, and let separate. Collect both TCFE solutions in second separatory funnel, add 20 mL water, shake, and let separate. Discard water (upper) layer. Add 20 mL water again and repeat washing. Transfer TCFE solution to 250 mL round-bottom flask and evaporate solvent under reduced pressure in rotary evaporator to 5–10 mL. Add 10 mL isopropanol and evaporate solvent to 1 mL.

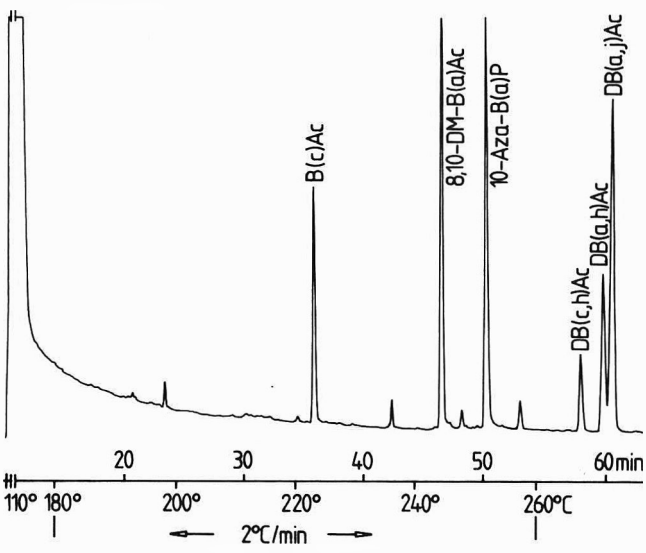

Figure 3. Gas chromatogram of reference solution (conditions as described).

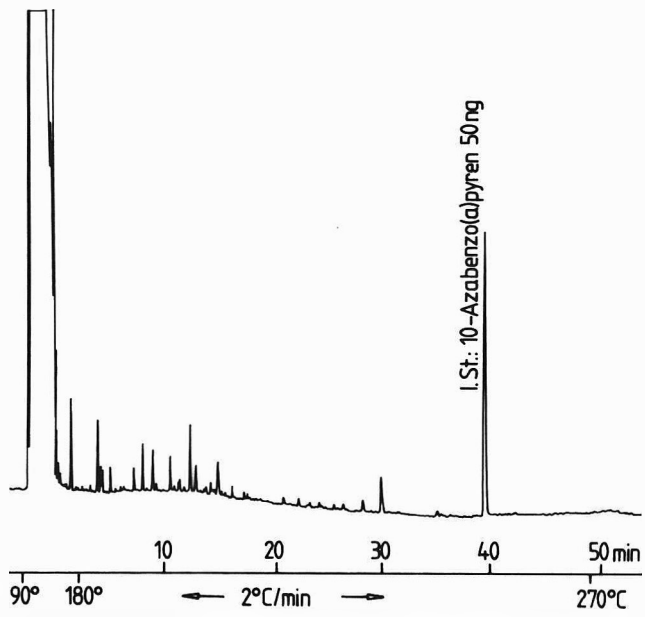

Figure 4. Gas chromatogram of solvent blank analysis with 10-azabenzo(a)pyrene as internal standard (GC conditions as described). Chromatography on Sephadex LH 20 was carried out in column as closed system.

Table 1. Recovery of various azaarenes related to 10-azabenzo(a)pyrene and to a second internal standard, dibenz(a,h)anthracene

	Related to 10-azabenzo (a)pyrene		Related to dibenz(a,h) anthracene	
Azaarene	ng	%	ng	%
Benz(c)acridine	339.8	103.9	263.7	86.7
10-Azabenzo(a)pyrene	int. std		438.4	83.1
Dibenz(c,h)acridine	152.8	103.9	118.6	85.4
Dibenz(a,h)acridine	383.5	102.3	297.6	83.4
Dibenz(a,j)acridine	783.9	100.1	608.3	82.6

Column Chromatography

Transfer solution (ca 1 mL isopropanol) to Sephadex LH 20 column and drain solvent until liquid is level with adsorbent. Rinse flask with 4 mL isopropanol and transfer rinse to column. Elute with 40 mL isopropanol; retain this eluate for analysis of N-PACs with less than 4 rings. Elute with additional 100 mL isopropanol and collect eluate in flask; this eluate contains N-PACs with more than 3 rings.

Preparation of Extract

Transfer 100 mL eluate in 2 portions to 100 mL round-bottom flask and evaporate almost to dryness in rotary evaporator under reduced pressure so as to maintain constant boiling (40°C water bath). Add 1 mL acetone to residue and transfer quantitatively to concentration tube (see Figure 2). Rinse 100 mL flask twice with 1 mL acetone and transfer rinse to concentration tube. Evaporate acetone almost to dryness in rotary evaporator, taking care to avoid splashes on walls of flask. Alternatively, evaporate solvent by blowing stream of purified nitrogen or by freeze drying. Rinse part of wall which is covered with solid residue, using 0.1 mL acetone, and repeat evaporation. Add 20 μL toluene–pyridine (99 + 1) and retain for gas chromatographic analysis.

Determination

Inject solution with 10 μL syringe at oven temperature of 110°C while split is closed. After 10 min, open split; after 1 min, increase temperature to 180°C at 20°/min, then to 260°C at 2°/min. Alternatively, use on-column injection at 100°C.

Following same procedure, inject N-PAC mixture to check shape of signals (peak tailing) and response factors of compounds. Under normal conditions (flame ionization detector well conditioned, no tailing), ratio of signal/weight is identical for all N-PACs present in mixture (Figure 3).

Calculate results by comparing peak areas of selected compounds to that of internal standard, 10-azabenzo(a)pyrene or 8,10-dimethylbenz(a)acridine.

Alternative to Liquid-Liquid Partition: Ion Exchanger

Liquid-liquid partition can be replaced by chromatography on an ion exchanger with no adsorption activity. This technique was not yet available for use in the collaborative study.

Dilute cyclohexane concentrate (1–3 mL) from liquid-liquid partition between DMF and cyclohexane with 3 mL methanol and transfer to column. Rinse flask with 3 mL methanol, and elute extract and rinse through column. Elute neutral compounds (PAHs, carbazoles, S-PACs) with 150 mL methanol. Remove basic compounds (N-PACs) from column with buffer solution: mixture of 30 mL 5N ammonium chloride, 10 mL 5N aqueous ammonia, 10 mL water, 50 mL methanol (total of 100 mL). Dilute eluted buffer solution with 100 mL 0.01N aqueous ammonia and extract with two 150 mL portions of cyclohexane. Wash cyclohexane solution with two 20 mL portions of 0.01N aqueous ammonia and evaporate to 10 mL. Add 30 mL benzene, evaporate solvent mixture again to 2 mL, transfer residue to smaller pointed flask (about 3 mL with ground glass joint), and concentrate to 0.2–0.3 mL as described above.

Table 2. Results of analysis (ng) of reference solution[a] (reference values in parentheses)

Lab.	BcAC (11.6)	8,10-D (23.1)	DBchAC (18.2)	DBahAC (21.1)	DBajAC (41.0)
1	15.7	22.9	(13.3)	(13.7)	(28.8)
	17.4	24.6	(22.6)	(11.9)	(23.1)
	16.2	23.0	(16.3)	(13.9)	(31.2)
2	10.4	(19.4)[b]	17.0	18.3	38.5
	11.0	(20.3)	16.9	18.8	39.0
	10.9	(20.0)	16.8	18.4	37.8
3	12.0	22.8	18.3	21.2	40.8
	11.8	22.8	18.3	20.8	41.0
	12.6	22.7	21.2	21.7	40.3
4	13.2	24.0	18.2	19.5	38.5
	13.2	23.6	18.6	20.1	40.4
	12.9	23.1	18.5	19.9	40.2
5	13.0	24.1	19.7	20.5	40.9
	13.9	25.2	19.5	20.2	42.0
	14.4	25.4	18.8	20.1	41.1
6	12.1	22.7	19.6	21.1	43.8
	11.9	22.3	19.2	20.7	43.0
	11.7	22.9	19.3	21.0	42.5
7[c](3)	13.0	24.6	19.7	21.3	44.9
Average	13.0	23.7	18.8	20.3	41.4
	(13.1)[d]	(23.1)	(18.0)	(19.2)	(39.3)
CV, %	13.6	4.0	5.5	5.2	5.8

[a]Internal standard: 10-azabenzo(a)pyrene, 24.6 ng/μg.
Acridines: benz(c)acridine (BcAC)
8,10-dibenz(a)acridine (8,10-D)
dibenz(c,h)acridine (DBchAC)
dibenz(a,h)acridine (DBahAC)
dibenz(a,j)acridine (DBajAC)

[b]Outlier.
[c]Means of 3 determinations.
[d]Average including outlier data.

Table 3. Results of analysis (μg/kg) of spiked meat samples[a] (spiked content in parentheses)

Lab.	BcAC (2.73)	DBchAC (1.23)	DBahAC (3.13)	DBajAC (6.53)
1	3.2	1.2	3.2	6.0
	2.7	0.6	2.4	6.6
$\bar{x}$	2.95	0.9	2.8	6.3
2	2.5	1.5	3.0	5.0
	2.1	1.3	3.1	5.5
$\bar{x}$	2.3	1.4	3.05	5.25
3	1.5	2.5	—	3.3
	0.6	0.9	—	2.9
$\bar{x}$	1.05	1.7	—	3.1
4	2.9	1.2	3.8	4.1
	3.0	1.3	4.1	4.9
$\bar{x}$	2.95	1.25	3.95	4.0
5	2.4	1.2	3.0	4.4
	2.8	1.2	3.3	9.9
$\bar{x}$	2.6	1.2	3.15	7.15
6	2.7	1.0	2.7	5.9
	3.6	0.9	2.3	5.6
$\bar{x}$	3.15	0.95	2.5	5.75
7	2.6	1.2	3.1	3.8
	2.4	1.1	3.2	5.3
$\bar{x}$	2.5	1.15	3.15	4.55
Average	2.7	1.2	3.1	5.2
CV, %	10.4	22.3	10.7	25.4

[a]Internal standard: 10-azabenzo(a)pyrene.

Results

The purity of all analytical materials used was checked by a solvent blank analysis (Figure 4).

The recovery of various azaarenes analyzed was determined by adding a second standard (dibenz(a,h)anthracene) immediately before GC analysis; recovery was 82.6–86.7% (Table 1).

Collaborative Study

Ten laboratories received a reference solution consisting of a mixture of 5 azaarenes (Figure 3) and 2 identical spiked ham samples for the determination of N-PACs. The composition of the reference solution is shown in Table 1. Participating laboratories were requested to compare the peak areas of the 5 compounds with the peak area of the internal standard, which corresponds to 24.6 ng/μL of 10-azabenzo(a)pyrene. Each laboratory was asked to repeat the analysis 3 times. In total, 19 analyses were received, but some values were rejected for statistical reasons. Outliers were determined according to Dean and Dixon (15) and Kaiser (16). These values are given in parentheses in Table 2. The coefficients of variation ranged from 4.0 to 13.6%.

Each laboratory received 2 identical samples of minced ham (40 g each), spiked with N-PACs in the ppm range. The results of 14 analyses are presented in Table 3.

Discussion

The key problem for analyses in the nanogram range is contamination by air-suspended matter in the laboratory during distillation of solvents and during enrichment procedures. Figures 5 and 6 show enrichment of the same sample with identical solvents, for a closed and open Sephadex LH 20 column, respectively. Contamination can be avoided by working under clean bench conditions and closed systems, avoiding contact with ambient air, as follows:

To avoid contamination of solvents, it is necessary to redistill them before use and to avoid contact of the distillate with laboratory air. In a closed system, the condenser has to be connected with a ground joint to the glass vessel, and the condensed solvent must be collected by pressure exchange through a small vent, protected by a filter. It is important to close the flask containing the redistilled solvent immediately after distillation.

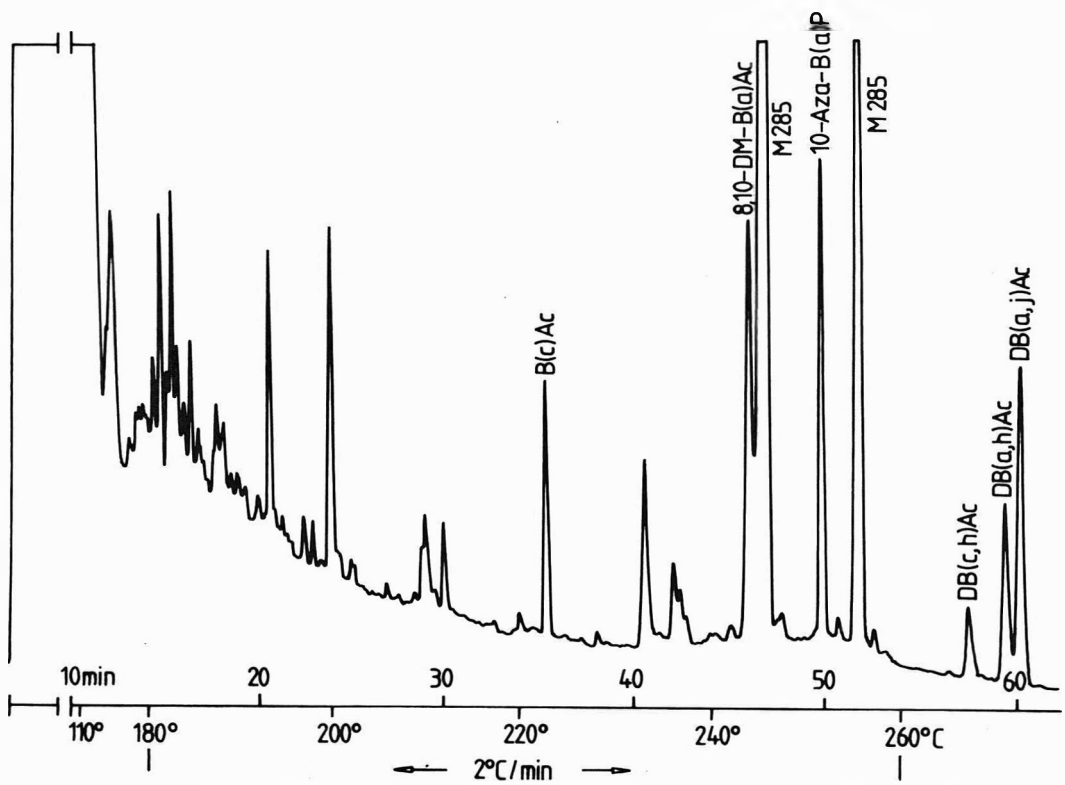

Figure 5. Gas chromatogram of N-PACs extracted from ham sample (conditions, see Figure 4).

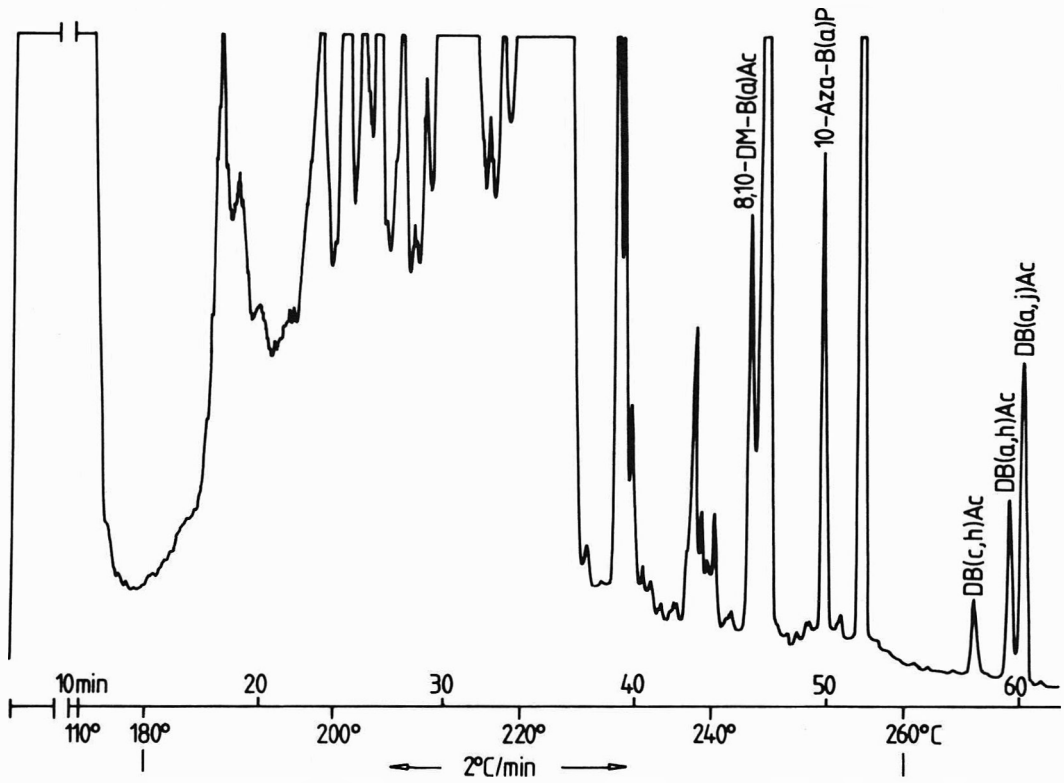

Figure 6. Gas chromatography of same ham sample as in Figure 5.
Chromatography on Sephadex LH 20 was carried out in column not protected against contamination from ambient air in laboratory (open system without connecting tube between solvent-input and output of the column).

To avoid contamination of glassware, it is necessary to rinse flasks and funnels with redistilled acetone and then with solvent used for the operation. Glassware must be protected from air with a ground glass stopper. During rotary evaporation, it is important to filter the air by a particle filter (e.g., glass fiber filter for air-suspended matter) when the vacuum is interrupted and air streams into the evaporator.

Basic azaarenes can be enriched specifically by extraction of cyclohexane solution with sulfuric acid, or preferably, by chromatography on an ion exchanger. The latter procedure is less dangerous, and avoids decomposition of some sensitive azaarenes such as benz(a)acridine.

In addition to the ham samples studied in the collaborative study, various other matrixes such as fresh and broiled meat as well as sausages were investigated. No measurable amounts of azaarenes were detected, although the added internal standard was recovered. A great number of azaarenes were found in petroleum (11), indicating that this method is suitable for analyzing other matrixes. Accordingly, IUPAC has decided to recommend this gas chromatographic method for determining basic nitrogen-containing aromatic compounds in high protein foods (17).

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Determination of Sulfite in Food by Flow Injection Analysis

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A method is described for the determination of sulfite levels in food products by flow injection analysis (FIA). The method is based on the decolorization of malachite green by SO_2, which is isolated from the flowing sample stream by means of a gas diffusion cell. The FIA method has a detection limit in food sample extracts of 0.1 ppm SO_2 (3 times peak height of blank), which corresponds to 1–10 ppm SO_2 in a food product, depending on the extraction procedure used. At the 5 ppm SO_2 level in a food extract, the precision of replicate injections is ± 1–2%. The method was tested on a variety of both sulfite-treated and untreated food products and the results compared favorably with those obtained by the Monier-Williams, colorimetric (pararosaniline), and enzymatic (sulfite oxidase) methods. The average differences from the FIA results were 19, 11, and 12%, respectively, for those samples (n = 12) above 50 ppm SO_2. At lower levels the results were somewhat more erratic due to inaccuracies of the various methods at low concentrations.

Sulfiting agents are used in a wide variety of different foods, mainly to control enzymatic browning. Although these substances have a long history of use, recent health-related problems in persons sensitive to sulfites have prompted renewed scrutiny by regulatory agencies for their presence in foods. In conjunction with this, it is desirable to determine low concentrations of sulfites, because the levels in many food products can be in the low ppm range. The currently available AOAC methods applicable to the quantitative determination of sulfites include the Monier-Williams and pararosaniline-based colorimetric methods (1). Although the Monier-Williams method is relatively sensitive and has been thoroughly collaborated (2), it involves a lengthy distillation and is tedious and time-consuming. An alternative official method based on the reaction between pararosaniline, formaldehyde, and sulfite has been developed (1), but it has limited sensitivity and involves an analytical scheme in which a number of variables have been shown to markedly affect color development (3). A method for sulfite determinations in foods is needed which is rapid, accurate, and sufficiently sensitive for low levels.

Flow injection analysis (FIA) is an analytical technique (4) that involves injection of a sample solution into a flowing stream. FIA techniques have been described for a wide variety of analytes in foods, including sulfites (5–7). One of the described procedures (5) is based on the pararosaniline reaction and uses a gas diffusion cell to separate evolved sulfur dioxide (SO_2) from the sample stream. Investigation of this method in our laboratory revealed both sensitivity and linearity problems that were likely due to a side reaction between formaldehyde and SO_2 (3). However, the application of FIA (with gas diffusion) to sulfite analysis appeared promising, and a number of other analytical strategies were tested, including the monitoring of pH changes caused by SO_2, the decolorization of permanganate, and the direct spectrophotometric measurement of SO_2 in the UV range. All of these techniques either lacked sufficient sensitivity or were subject to interferences. However, a procedure based on decolorization of malachite green proved to be an effective means of measuring sulfite by FIA, and a method based on this reaction was developed. This paper describes the construction and application of a flow injection analyzer for the determination of sulfite in food extracts and a comparison of the results of the FIA method to those of the Monier-Williams, colorimetric, and enzymatic methods for a number of different food products. The purpose of this study was to evaluate the final determinative step. Extraction efficiency of residual sulfite and recovery of added sulfite were not studied.

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METHODS

Reagents

(a) *Concentrated sodium tetrachloromercurate.*—Dissolve 23.4 g NaCl and 54.3 g $HgCl_2$ in ca 1900 mL water and dilute to 2 L (sec. **20.126**(c)) (1).

(b) *Sodium tetrachloromercurate.*—Dissolve 4.7 g NaCl and 10.9 g $HgCl_2$ in ca 1900 mL water and dilute to 2 L (sec. **20.044**(b)) (1).

(c) *Stock phosphate-buffered (0.0625M) malachite green.*—200 ppm. Dissolve 200 mg malachite green (99%, No. C1264, Eastman Organic Chemicals, 343 State St, Rochester, NY 14650), and 8.5 g KH_2PO_4 in ca 900 mL water and dilute to 1 L. Filter prepared solution through 0.45 μm cellulose acetate membrane filter and store at 4°C.

(d) *Dilute malachite green reagent.*—Dilute stock solution (c) 1 + 9 with water. Prepare fresh daily.

(e) *Phosphate buffer.*—0.094M. Dissolve 16.36 g K_2HPO_4 in ca 900 mL water and dilute to 1 L.

(f) *FIA donor reagent.*—0.15M H_2SO_4. Add 8.3 mL concentrated H_2SO_4 to ca 900 mL water and dilute to 1 L.

(g) *Sulfite stock standard.*—500 ppm SO_2. Dissolve 98.4 mg Na_2SO_3 in ca 90 mL tetrachloromercurate solution (b) and dilute to 100 mL.

(h) *Sulfite working standards.*—0–20 ppm. Dilute stock standard (g) with tetrachloromercurate reagent (b) to obtain standards equivalent to 20, 15, 10, 8, 6, 4, 2, 1, and 0 ppm SO_2.

(i) *FIA rinsing solution.*—0.02M NaOH. Dissolve 0.8 g NaOH in 1 L water.

(j) *Acetaldehyde preserved stock standard.*—500 ppm SO_2. Prepare as in (g), except replace tetrachloromercurate solution with 1% acetaldehyde.

Apparatus

Flow injection analyzer.—Construct FIA apparatus as shown in Figure 1, consisting of peristaltic pump providing relatively pulse-free operation (Polystaltic, Buchler Instruments, Fort Lee, NJ, or equivalent), liquid chromatography loop-type injection valve equipped with 50 μL loop (Valco Inc., or equivalent), low volume gas diffusion cell (8200-0200, Control Equipment Corp., 171 Lincoln St, Lowell, MA 01852) equipped with Teflon membrane (8200-0201), variable wavelength UV-vis detector (Spectra-Physics, Model 770, or equivalent), and chart recorder (Omniscribe, Fisher Scientific, or equivalent). Install 3 equal diameter pump tubes (ca 0.8 mm id, acid-resistant) on pump, and using variable speed control, determine setting that provides 0.75 mL/min flow for each line. Construct remainder of FIA manifold as shown in Figure 1 with Teflon tubing and low volume fittings, taking care to minimize dead volumes wherever possible. Remove narrow bore stainless steel lines leading to detector flow cell and

Table 1. Results of SO_2 determination by flow injection analysis, Monier-Williams, colorimetric, and enzymatic methods

Sample	Product	Sulfite treatment, % used	Sulfite, ppm			
			FIA	M-W	Color.	Enzym.
1	white wine	A[a]	92	165	105	93
2	red wine	A	49	91	70	112
3	apple juice	0	ND(1.5)[b]	NS(7)[c]	NS(5)[c]	NS(5)[c]
4	apple juice	0.01[d]	88	87	104	72
5	apple juice	0.05[d]	478	448	493	420
6	guacamole	0	ND(10)	NS(11)	NS(33)	ND(33)
7	guacamole	0.05[e]	653	494	785	558
8	dried apricots	A	1710	2043	2010	1680
9	potatoes	0	ND(6)	ND(16)	NS(20)	NS(20)
10	potatoes	0.4[f]	284	263	295	210
11	potatoes	2.0[f]	1544	1492	2104	1338
12	pickled onions	A	13	36	14	22
13	Gulf shrimp	0	ND(2)	NS(11)	NS(5)	ND(5)
14	Gulf shrimp	1.25[g]	192	131	204	174
15	Gulf shrimp	5.0[g]	603	496	623	632
16	shrimp	0	ND(2)	NS(11)	NS(5)	ND(5)
17	shrimp	0	ND(2)	NS(6)	NS(5)	ND(5)
18	shrimp	0	ND(2)	NS(6)	NS(5)	NS(5)
19	shrimp	0	ND(2)	NS(6)	NS(5)	NS(5)
20	dried apples	A	1694	2091	1764	1744
21	lettuce	0	ND(10)	NS(11)	NS(25)	ND(25)
22	lettuce	0.4[f]	465	417	463	311
23	cabbage	0	ND(10)	NS(11)	NS(26)	ND(25)
24	cabbage	0.4[f]	193	184	176	187

[a]Sulfiting agents added during processing.
[b]Not detected (ND); detection limit given in parentheses.
[c]Not significant (NS); positive response was detected that was below the specified detection limit of the method. Detection limit given in parentheses.
[d]Na_2SO_3 added directly to juice at stated level.
[e]Commercial sulfiting agent added at stated level.
[f]1 min dip in solution of commercial sulfiting agent.
[g]1 min dip in solution of $NaHSO_3$.

replace with Teflon tubing (0.4 mm id) to minimize back pressure. Mount gas diffusion cell vertically with flow in an upward direction to facilitate purging of air bubbles. After system is completely assembled, check flow rate of reagents through detector to ensure that all restrictions have been removed and total flow is ca 1.5 mL/min.

Sample Treatment

Commercially available dry mix containing ca 60% available SO_2 (w/w), citric acid, sodium bicarbonate, and ascorbic acid was used to treat the guacamole, potatoes, lettuce, and cabbage. Analytical grade sodium bisulfite and sodium sulfite were used to treat the shrimp and apple juice, respectively. Either the products were dipped in a solution of the sulfiting agents (potatoes, lettuce, cabbage, and shrimp) or sulfiting agent was added directly to product (apple juice and guacamole) at levels shown in Table 1.

All treated samples and untreated controls were stored before extraction for 16 h at 4°C in covered glass containers. Dried apples, domestic and imported shrimp, and pickled onions were received in the Seattle District Laboratory of the Food and Drug Administration (FDA) for analysis. Dried apricots, white wine, and red wine were purchased at a local retail store.

Extraction Procedures for FIA and Colorimetric Methods

All samples were composited and extracted as described in an established AOAC method (sec. 20.128). Vegetable- and fruit-based products (40–50 g) were extracted with 4 volumes of water. A 5–25 mL portion of extract was treated with base to free bound sulfite (8), neutralized, preserved with concentrated tetrachloromercurate reagent (a) (at 20% of final volume), and diluted to volume (5–200 × sample weight, depending on SO_2 content) with water. Just before analysis, extracts were centrifuged (8000 × *g*) for 10 min and diluted with tetrachloromercurate reagent (b) to within analytical range (0–20 ppm SO_2).

Shrimp samples (40–50 g) were extracted with dilute tetrachloromercurate reagent (b) so that final dilution was 1 g shrimp per 5 g final weight. Just before analysis, samples were centrifuged (8000 × *g*) for 10 min and diluted to within analytical range (0–20 ppm SO_2) with tetrachloromercurate reagent (b). All extracts were analyzed either immediately after extraction or stored at 4°C for maximum of 24 h.

Extraction Procedure for Enzymatic Method

For enzymatic analysis, samples were extracted in 4 volumes of water, centrifuged (8000 × *g*), and treated as in the described method (9). All vegetable- and fruit-based products were treated with ascorbic oxidase before analysis as described (9).

Determinations

FIA method.—Establish flow (0.75 mL/min each) of FIA donor reagent (f), dilute malachite green reagent (d), and phosphate buffer (e) through lines A, B, and C (Figure 1), respectively. Monitor absorbance of solution flowing through detector at 615 nm and wait for establishment of stable baseline (ca 10 min). Set detector sensitivity to 0.2 AUFS and adjust pen on recorder to 90% full scale. Inject 20 ppm standard (50 μL) repeatedly until consistent peak height is achieved (± 2%). (Note that negative peaks are produced as malachite green is decolorized). Adjust sensitivity of detector so that 20 ppm SO_2 standard produces ca 90% full scale peak. Once system has stabilized, inject standards (0–20 ppm) and samples (diluted to within range of standards with tetrachloromercurate reagent (b)) in duplicate.

Determine concentration of SO_2 in sample extracts by comparing peak heights with standard curve (ppm SO_2 vs peak height). Calculate SO_2 content in food product by multiplying extract concentration by dilution factors relevant to extraction procedure used.

After last sample has been injected, wash out FIA system by pumping ca 20 mL water followed by ca 20 mL 0.02M

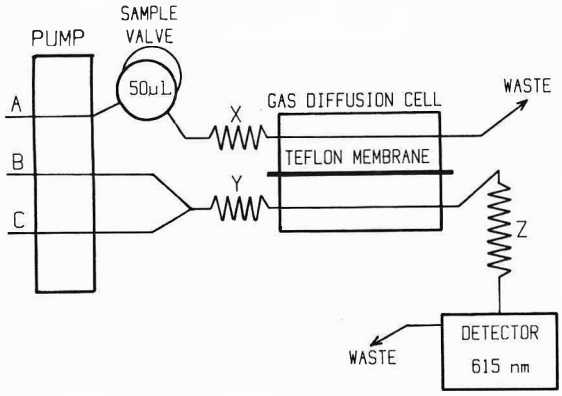

Figure 1. Flow diagram for flow injection analyzer. Reagents: A, 0.15M H_2SO_4; B, dilute malachite green reagent; C, phosphate buffer. Mixing coils: X (0.5 mm id × 60 cm); Y (0.5 mm id × 60 cm); Z (0.5 mm id × 260 cm).

NaOH (i) through each line. Apparatus can be left with NaOH solution in lines. Release pump tube roller pressure when not in use.

Colorimetric method.—Samples were analyzed as described for AOAC colorimetric method (sec. **20.128**) (1) with following modifications. All reagent and sample volumes were decreased proportionally and reaction was carried out in square polystyrene disposable cuvets (Ultra-Vu, American Scientific Products, 1430 Waukegan Rd, McGaw Park, IL 60085) with final reaction volume of 3.4 mL. A sample blank was prepared for every sample in which pararosaniline reagent was replaced with 0.95N HCl (80 mL concentrated HCl/L water). Absorbance of blank was subtracted from each corresponding sample to determine net absorbance due to SO_2 in sample.

Enzymatic method.—SO_2 content in sample extracts was determined as in instructions contained in the Sulfite Test Kit (No. 725854, Boehringer Mannheim Biochemicals, 7941 Castleway Dr, PO Box 50816, Indianapolis, IN 46250) (9). SO_2 standards (3 and 10 ppm), prepared from acetaldehyde-preserved stock standard (j), were included with every batch of samples to check performance of test kit procedure. Recovery of SO_2 from standards was generally 85–95%.

Monier-Williams method.—SO_2 content in 5–50 g (depending on level) of composited food sample was determined as in AOAC Monier-Williams method (secs **20.123–20.125**) (1), using titrimetric method to determine SO_2 evolved. Blank titration volume depended on freshness of H_2O_2 used, so a blank was analyzed every 3–4 days and sample titration volumes were adjusted accordingly.

Results and Discussion

Flow Injection Analysis

A number of different FIA configurations were tested for the determination of sulfite, but the system based on decolorization of malachite green produced the best results in terms of linearity, sensitivity, and freedom from interferences. In this system, SO_2 generated in the donor stream diffuses across the Teflon membrane in the gas diffusion cell and reacts with the central carbon atom of malachite green (10). Because malachite green is unstable at elevated pH (decolorizes) and the reaction with sulfite occurs most rapidly around pH 8, it is necessary to generate the malachite green reagent on-line, just before it enters the diffusion cell. The recipient stream in the gas diffusion cell consists of a 10 ppm malachite green solution buffered at pH 8.0 with 0.05M potassium phosphate after the 2 recipient reagents (B and C) are mixed in equal proportions in the Y mixing coil (Figure 1).

A number of variables affected the sensitivity of the system for detection of SO_2, with the flow rate of the 2 reagents passing through the diffusion cell perhaps being most important. The sensitivity of the system can be improved markedly by decreasing the total flow through the cell, thus allowing more time for diffusion of SO_2. Decreasing the flow rate of each reagent line from 0.75 to approximately 0.2 mL/min produces a 3- to 4-fold increase in peak height (peak width also increases), allowing low level SO_2 determinations. At the lower flow rate, the detection limit of the system is approximately 0.1 ppm SO_2 (3 × baseline noise), which corresponds to 1–10 ppm SO_2 in the food product. Since the frequency at which samples can be injected decreases and the levels of sulfite in extracts of most food products are in the 0–20 ppm range, operation of the FIA system with the flow rate of each reagent line at 0.75 mL/min provides a good compromise between sensitivity and speed and provides a detection limit of approximately 0.3 ppm SO_2 in the extract.

In addition to the total reagent flow through the gas diffusion cell, the relative flow rates of the donor and recipient streams affect sensitivity. Diffusion of SO_2 across the membrane is optimized by operating the cell with the donor stream (H_2SO_4) flowing at a much lower flow rate than the recipient stream. Like total flow rate, the system as described here, with the donor flow at one half the velocity of the recipient, provides a good compromise between sensitivity and speed.

The FIA system provides a rapid, sensitive means for the determination of sulfite in food sample extracts. Figure 2 illustrates a typical series of injections of samples and standards. Since the time between injections is short (about 60 s), it is convenient to analyze all samples and standards in replicate, thereby increasing accuracy. Once the system has stabilized, which generally takes about 10 min, the precision in a series of replicate injections is generally ± 1–2% at the 5 ppm SO_2 level. The system is linear in the 0–20 ppm range and recovery of SO_2 in a number of sample extracts spiked at 2 and 10 ppm averaged 96%. This recovery value is for SO_2 added to stabilized shrimp extracts and does not reflect the recovery of SO_2 from foods spiked either before or during the extraction process, which may be lower (unpublished data).

Extracts of untreated food samples (Nos. 3, 6, 9; Figure 2) and the standard blank exhibit no peaks, indicating a general lack of nonspecific interferences in the FIA system described here. For a substance to be a potential interferent in the system, it must have a relatively high vapor pressure under the conditions existing on the donor side of the Teflon membrane (acidic pH, room temperature) so that transport across the membrane can occur. Additionally, it also must interfere with the reaction between malachite green and SO_2. A number of potential interfering substances were tested including cyanide, sulfide, carbonate, hypochlorite, thiosulfate, thiocyanate, and short-chain fatty acids (C_1 and C_4). Of these, none produced negative interferences (i.e., decreased the response of a 10 ppm SO_2 standard when added at the level of 500 ppm) except hypochlorite (which was probably due to oxidation of sulfite to sulfate). Only sulfide and cyanide produced peaks (positive interferences) in the system. For both of these interfering compounds, the response was less than SO_2. Approximately 7 ppm sulfide and 750 ppm cyanide were necessary to produce a response equivalent to 2 ppm SO_2. Although the variety of food products tested so far on the FIA system is not great, it would be expected that the only products exhibiting significant interferences would be those

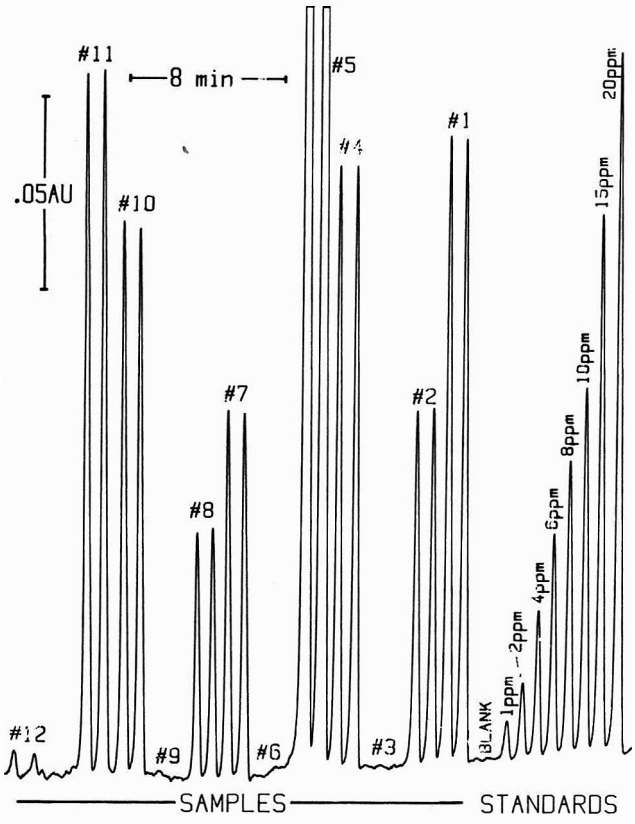

Figure 2. Chart recording from FIA system for analysis of SO_2 in sample extracts 1–12 (Table 1). Standards given in ppm SO_2. All samples injected in duplicate.

with volatile sulfur compounds such as onions, garlic, and cabbage. Further testing will be necessary to establish the range of products for which the FIA system is applicable.

In addition to detecting the malachite green-SO_2 adduct by monitoring the decrease in absorbance at 615 nm, it is also possible to monitor increasing absorbance at 254 nm, since there is a shift in the absorption spectrum of malachite green on addition of SO_2 (11). Although this mode of detection has not been tested thoroughly, the sensitivity is approximately the same as at 615 nm and may prove to be a viable alternative if a detector capable of being operated at 615 nm is not available.

The apparatus, as described here, proved to be very effective in determining the level of sulfite in food extracts which were stabilized with tetrachloromercurate. These sample extracts had a low buffering capacity, and therefore 0.15M H_2SO_4 as the donor reagent was sufficient to maintain a very low pH on the donor side of the membrane. In sample extracts with substantial buffering capacity, it may be necessary to increase the strength of the donor acid to 1M to maintain the proper pH.

Comparison of Methods

To determine the applicability of the FIA system to the determination of sulfite levels in foods, a number of samples representing a variety of food products were analyzed by several different methods. These included the Monier-Williams method involving distillation of SO_2 into H_2O_2 with determination of H_2SO_4 formed by titration, the colorimetric method based on pararosaniline, and an enzymatic-based method using sulfite oxidase and NADH-peroxidase. The study was not designed to investigate efficiency of recovery of bound and/or free SO_2 from samples, but only to evaluate the final determinative steps. Therefore, wherever possible, standard AOAC sample preparation and extraction techniques were used.

Examination of the data (Table 1) reveals that the results of the 4 analyses agree quite well. The average differences from the FIA results were 19, 11, and 12% for the Monier-Williams, colorimetric, and enzymatic analyses, respectively, in all samples over 50 ppm SO_2 (n = 12). Below this level, the results show a wider degree of variation, due in part to inaccuracies inherent in any method at levels approaching the detection limit. Since the sample preparation procedures were quite different for the various methods (distillation for Monier-Williams vs extraction for the other methods), it is likely that much of the variation observed in the results of the different methods is due to differences in the efficiency of recovering bound sulfite. This question was not addressed in the present study, but it is likely that a better correlation could be achieved by modification of the extraction procedures to more effectively recover bound residues of SO_2. In general, the FIA procedure is more sensitive than the other 3 methods tested, although in the case of the Monier-Williams method it may be possible to lower the detection limit by using a larger sample weight.

Since a primary goal of this study was to determine whether some food products might produce false positives in the FIA method, a number of samples were included which had no previous sulfite treatment, and for these samples no positive interferences were detected by FIA. To calculate the lower limit of detection by the various methods, a series of reagent blank determinations were made. The "detection limit" was then set at a value 3 times the range for the blank determinations and is reported in parentheses in Table 1. Note that this value varies depending on the absolute detection limit of the method and the dilutions that were made during the sample extraction procedure (or in the case of the Monier-Williams method, the weight of sample used). In Table 1, 2 categories of samples are identified that fell below the calculated detection limit. "ND" designates those that gave a response equal to the blank; "NS" designates those that produced a positive response, but at a level deemed to be not significant (i.e., below 3 times the range of the blanks). It is interesting to note that low level positive responses (i.e., "NS") below the detection limit occurred in many samples by all except the FIA method (see Table 1).

Conclusions

In general, the results of these investigations reveal that the FIA procedure is a viable technique for the rapid, accurate determination of sulfite levels in a variety of foods. The method is generally free of interferences and the results correlate well with those by established methods. Among the advantages of the FIA method over the other available methods are the following:

(1) FIA is extremely rapid, allowing the SO_2 content of an extract to be determined in approximately 60 s.
(2) The instrumentation is easily automated.
(3) For those food products tested, FIA appears to be less subject to interferences than any of the other methods.
(4) FIA is more sensitive, with a lower detection limit of approximately 0.1 ppm SO_2 in a food extract.
(5) The instrumentation is relatively simple and inexpensive.
(6) Highly turbid or pigmented samples can be injected with no sample cleanup.

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SEAFOOD TOXINS

Variability of Mouse Bioassay for Determination of Paralytic Shellfish Poisoning Toxins

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Toxic shellfish extracts and paralytic shellfish poison (PSP) standard solutions, tested over a range of pH levels, storage conditions, and temperatures, were monitored for toxin concentration, using the mouse bioassay and thin layer chromatography (TLC). A comparison of PSP toxin concentrations in toxic shellfish extracts and PSP standard solutions when dilution was varied suggests that other factors in the shellfish extracts contribute to the toxicity in mice; the closest agreement was at the death time range of 5–8 min. The toxicities of PSP standard solutions at pH levels ranging from 2 to 6 and held at 4°C for various times were relatively constant; however, there was a gradual decrease in toxicity with pH 6 solutions. Also, standard solutions (pH 6) held at 4°C for 28 days showed a 50% decrease in toxicity when the pH was adjusted to 2. TLC analyses of PSP standard solutions and toxic shellfish extracts revealed multiple spots at the R_f ranges of saxitoxin/neosaxitoxin and gonyaulax toxins I–IV. PSP standard solutions usually had a single spot in the saxitoxin/neosaxitoxin area. No attempt was made to confirm the identity of these compounds. Previously tested toxic shellfish extracts with subsequent pH adjustment to 1.5 and additional heat treatment (100°C for 5 min) showed no appreciable difference in mouse toxicity. The use of antifoaming agents during the acid extraction step did not affect the final amounts of PSP obtained.

Paralytic shellfish poisoning (PSP) in humans has been recorded from various parts of the world for centuries. An excellent comprehensive review on environmental health criteria for marine and freshwater biotoxins has been prepared by the World Health Organization (1). PSP is caused by ingestion of shellfish (oysters, clams, and mussels) that have fed on toxigenic dinoflagellates such as *Protogonyaulax tamerensis* or *P. catanella*. Many other seafoods and marine organisms have also been implicated in PSP. Twelve structurally different toxins associated with PSP have been structurally identified (2). Saxitoxin, neosaxitoxin, and the 4 epimeric 11-hydroxysulfate esters (gonyaulax toxins I–IV) appear to have the highest toxicity for the mouse; however, Hall (3) showed that several of the carbamoyl-*N*-sulfo derivatives of saxitoxin and neosaxitoxin that had low toxicities in the initial extract had significantly increased toxicity following hydrolysis (about 0.2M HCl, 100°C, 5 min). On the other hand, Quayle and Bourne (4) reported that toxic butter clams, boiled under a range of low pH levels (1.8–4.9) for varying times (2.5–10 min), did not show any significant differences in toxicity to the mouse. They also reported that normal cooking and canning operations reduced the toxin content by 90% when analyzed by the AOAC procedure (5).

The mouse bioassay (5), adopted as an official AOAC method in 1965, remains the accepted procedure for detecting and measuring the toxins in shellfish. Toxin content is expressed as μg PSP/100 g shellfish meat when the mouse response is standardized against a PSP standard solution (saxitoxin, >95% purity). Test conditions, such as animal strain and sex (6, 7), salt concentration (8), and sample preparation (9), significantly affect test results. In addition, the potential for foaming during the acid extract boiling phase requires constant surveillance of the samples. The following study was undertaken to provide additional information on effect of pH, time, and sample treatment on the toxicity observed using the standard mouse assay.

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Experimental

Mouse Bioassay

The standard mouse bioassay (5) was used for the detection and quantitation of PSP toxins.

Thin Layer Chromatography (TLC)

Samples subjected to time and pH stability studies were analyzed according to the procedures of Buckley et al. (10) as modified by Hall (3). EM silica gel 60 F-254 aluminum-backed TLC plates were used. The developing solvent was pyridine–acetic acid–water–ethyl acetate (45 + 9 + 12 + 15); the pyridine–acetic acid–water (225 + 45 + 60) was prepared as a stable premix and mixed with ethyl acetate immediately before development of the TLC plate (premix–ethyl acetate (66 + 15)). Following development, the plates were allowed to air-dry, and then sprayed with 1% H_2O_2 solution, heated to 120°C for 15 min, and scanned under 366 nm UV light for fluorescent spots. Before TLC analysis, aqueous samples were freeze-dried, redissolved in 150 μL water, and transferred to 1 dram vials. Methanol (100 μL) was added to each sample and 50 μL was spotted using a Camag Linomat III automatic TLC spotting system.

PSP Toxins

Standard PSP toxin solutions (saxitoxin, >95% purity) were obtained from the Food and Drug Administration, Division of Microbiology, Cincinnati, OH 45202. Working solutions for the pH variation experiment were prepared by diluting the standard solution with water to concentrations of 0.1, 0.25, 0.33, 0.5, 0.67, 1.0, 2.0, and 5.0 μg PSP/mL and, where indicated, the pH was adjusted with 0.1N HCl. For the dilution effect experiment, 4 μg PSP/mL and 800 μg PSP/100 g shellfish meat (equivalent to 4 μg/mL extract) solutions were prepared and analyzed for PSP toxin concentrations at subsequent dilution levels.

Shellfish Meat Extracts

Samples of naturally incurred toxic and nontoxic shellfish meats (mussels *(Mytilus edulis)* and clams *(Mya arenaria)*) were collected by the Maine Department of Marine Resources off the Maine seacoast. PSP toxins were extracted from the homogenized meat according to the standard AOAC mouse bioassay (5) on the day of sample collection and stored at 4°C until completion of analysis.

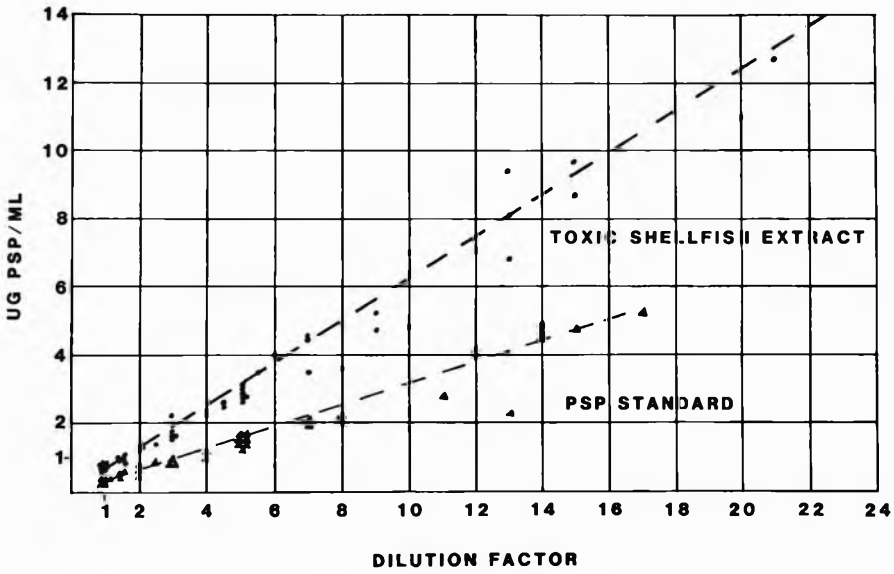

Figure 1. Comparison of PSP concentration in PSP standard solutions and toxic shellfish extracts as determined by mouse bioassay starting with samples containing different concentrations of PSP.

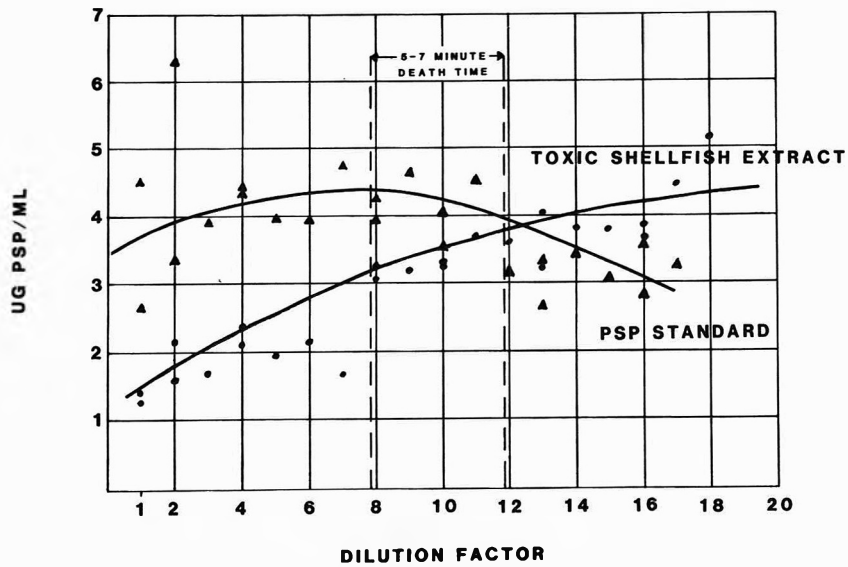

Figure 2. Effect of dilution factor on PSP concentration in PSP standard solutions and toxic shellfish extracts as determined by mouse bioassay with the PSP concentration determined at each dilution by ip mouse injection.

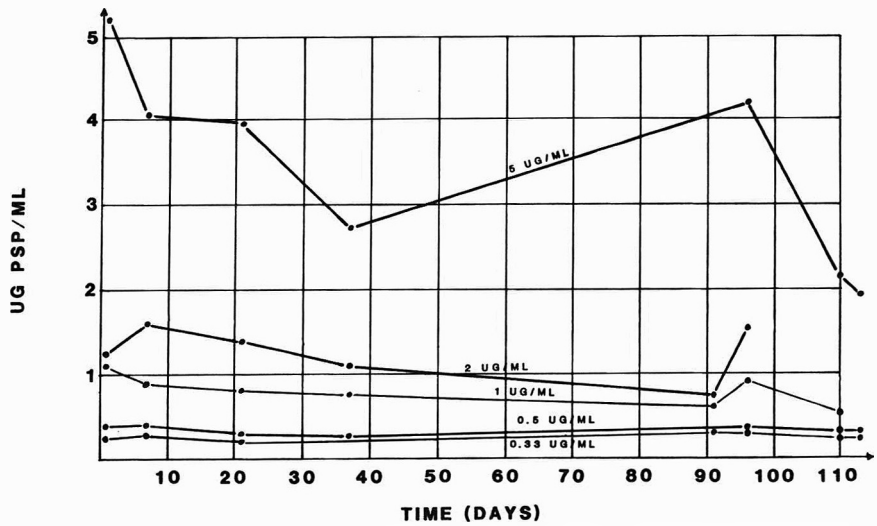

Figure 3. Relative changes in concentration of PSP standard solutions at ca pH 6.0 stored at 4°C. Initial concentrations were 5.0, 2.0, 1.0, and 0.33 μg PSP/mL.

Table 1. Relative concentration (μg/mL) of PSP standard solutions as determined by mouse bioassay with varied pH and time

Calcd PSP std concn, μg/mL	pH						Time, days			
	Day 0			Day 31						
	2.7	4.3	6.0	2.7	4.3	6.0	0	14	28[a]	35[a]
1.0	1.45	1.40	1.26	1.33	1.28	1.50				
2.0	2.46	2.50	2.15	2.80	2.29	2.08	1.71	1.45	0.67	—[b]
5.0	5.24	4.14	5.12	5.38	5.69	4.50	4.69	4.56	1.53	1.56

[a]pH adjusted to 2.0.
[b]Not tested.

Table 2. Relative concentration (μg/100 g shellfish meat) of saxitoxin in toxic shellfish extract before and after pH adjustment to 1.5 and additional boiling (100°C) for 5 min

AOAC method (5) (pH 2.4)	pH 1.5 plus boiling	Diff., %
Mytilus edulis		
301	301	0
611	532	− 12.9
989	1101	+ 11.3
1260	1281	+ 1.7
1339	1572	+ 17.4
Av. 900	957	+ 6.3
Mya arenaria		
40	36	− 10.0
75	68	− 9.3
102	100	− 2.0
248	268	+ 8.1
277	244	− 11.9
534	471	− 11.8
Av. 213	198	− 2.3

Determination of pH

The pH of PSP standard and toxic shellfish extract solutions was determined using an Accumet Model 620 pH meter (Fisher Scientific Co.) with appropriate reference buffer solutions.

Antifoaming Agents

Three commercially available antifoaming agents (Dow Corning C and H-10, and Anti-foam B (Baker grade)) were added to the acid extract of toxic mussels to prevent boiling over of the extract, and the extracts were then analyzed as outlined in the AOAC method (5). Three concentrations of the antifoaming agents (5, 50, and 150 ppm) were tested in the toxic shellfish meat, and separate solutions at concentrations of 150 ppm were used as controls.

Results and Discussion

Figures 1 and 2 show the relationship between the concentration of PSP toxins in toxic shellfish extracts and PSP standard solutions and the dilution factor. Figure 1 shows the dilution factor required to obtain a 5–7 min death time starting with samples containing different concentrations of PSP. Figure 2 shows subsequent dilutions of standard and toxic shellfish extracts with the PSP concentration determined at each dilution by intraperitoneal (ip) mouse injection. These results suggest that other factors influence the toxicity in mice of the shellfish extracts, since the results show that the dilution factor for the standard is approximately double that of the extract at the same concentration. The salt effect previously reported (8) may account for the variability at lower dilutions; however, its effect should be minimal at higher dilutions. The agreement between standard solutions and shellfish extracts would be expected at higher dilutions. The closest agreement was between the 5–8 min death time range (8–14 dilution factor, Figure 2). A comparison of the mouse units vs dilution factor reveals a wider scattering of the data points for the shellfish extracts, but no definite trend was evident.

Several PSP standard solutions with varied pH and holding times at 4°C were monitored for toxicity using the mouse bioassay. Relatively consistent results were observed with time regardless of the pH of the test solution (Table 1). Figure 3, however, shows the gradual decrease in test values obtained with time at pH 6.0. No definite pattern was observed, emphasizing the variability of the method itself or suggesting chemical transformation of the toxins or the presence of other compounds that affected the results. This is particularly important when samples are stored or transported to other locations before analysis. Standard solutions at pH 6 held at 4°C for 28 days showed a marked reduction in toxicity when the pH was adjusted to 2 (Table 1). TLC analysis of PSP standard solutions held between 30 and 60 days usually revealed a single spot at the same R_f as saxitoxin and neosaxitoxin. However, toxic shellfish samples and some freshly prepared PSP standard solutions showed additional fluorescent spots in the range of the gonyaulax toxins I–IV (R_f 0.80–0.82). The identity of these compounds was not confirmed and should be investigated further. All samples showing multiple spots had an original PSP concentration >2 μg/mL. The samples exhibiting a single spot may have also contained additional fluorescent compound(s) but at levels below the detection limit of the TLC method. No attempt was made to quantitate or confirm the identity of these compounds.

Extracts from selected toxic shellfish (*Mytilus edulis* and *Mya arenaria*) obtained off the Maine seacoast were tested further by adjusting the pH to about 1.5 and heating to 100°C for 5 min to evaluate the potential hydrolysis of the carbamoyl-*N*-sulfo derivatives. Toxin profile studies (D. L. Park, unpublished data, 1985) have shown the presence of these toxins in shellfish extracts from Maine seacoast areas. No appreciable difference in mouse toxicity was observed (Table 2), in agreement with results reported by Quayle and Bourne (4). The use of antifoaming agents during the acid extraction step did not affect the final levels of PSP obtained (Table 3). All antifoaming agents substantially reduced foaming at the levels tested, and it is suggested that the AOAC method be revised to allow the use of these products.

The pH of the shellfish extracts (toxic and nontoxic) when analyzed by the AOAC method averaged 4.4 with a range of 3.3–5.9 over a period of several months. The authors suggest that the AOAC method be revised to clearly outline the pH adjustment procedure and the need to confirm the proper pH strongly emphasized. Analysts relying on the mouse bioassay to determine PSP toxin levels in shellfish should also be aware of the inherent variability of the method and the potential for underestimating the actual toxin levels (Figures 1 and 2). Several toxins with varied toxicities are associated with PSP outbreaks (2, 3, 10) and the predominance or chemical transformation of these toxins can be influenced by many factors,

Table 3. PSP concentration[a] (μg/100 g shellfish meat) in mussels (*Mytilus edulis*) as determined by mouse bioassay (5) with/without use of antifoaming agent during acid extraction

	Antifoaming agent		
Concn, ppm	Dow Corning C	Dow Corning H-10	Anti-foam B
0		179/148	
5	179/169	165/165	189/183
50	146/179	165/186	165/165
150	160/136	173/165	141/160
Control	—[b]	—[b]	—[b]

[a]Duplicate analyses.
[b]No toxic effect observed.

including sample preparation, pH, and storage. The preparation and purity of PSP standards will also influence the accuracy of the method.

Acknowledgment

The authors thank John Hurst, Maine Department of Marine Resources, West Boothbay Harbor, ME, for supplying toxic shellfish extracts.

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FRUIT AND FRUIT PRODUCTS

Gas Chromatographic Determination of Fatty Acids and Sterols in Orange Juice

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A gas chromatographic (GC) method has been developed for the simultaneous quantitation of fatty acids and sterols in orange juice, using a bonded phase fused silica capillary column of intermediate polarity, splitless automatic injection, and flame ionization detection. Sample preparation has been simplified by using 1 g C-18 adsorbent in a disposable minicolumn to extract 2 mL orange juice. Methylation of fatty acids and silylation of the sterols were carried out in the eluted extract (low polarity lipid fraction). The method precision was 7%; recoveries ranged from 83 to 113%. The precision of the injection technique was 2%. Seven major fatty acids and 5 sterols in orange juice were quantitated by the GC method and identified by GC/mass spectrometry. Quantitative data for several orange juice samples indicated that the levels of the compounds of interest were in the 1.3–72.0 mg/L range. The results demonstrate that bonded phase fused silica capillary GC has great versatility and potential for the quantitative determination of fatty acids and sterols.

Procedures used for determining lipids in citrus juice can reveal important information about the chemical characteristics of the product. For example, these methods have shown that citrus species may be differentiated by their lipid composition (1), storage time affects the lipid composition of orange juice (2), and closely related orange cultivars have different lipid profiles (3). In addition, lipid profiles may be important in the detection of orange and grapefruit juice adulteration by using pattern recognition, a mathematical and statistical approach to the treatment of the compositional data (4). Compositional studies of this kind require the analysis of a large number of samples, and therefore, simple and rapid methods are needed.

The methods used for the determination of fatty acids and sterols in citrus juices have consisted mainly of extraction with organic solvents, separation on Sephadex or Celite columns, and thin layer chromatography or packed column gas chromatography (GC) with flame ionization detection. These methods have required large test portions and solvent volumes. On the other hand, fused silica capillary GC, with its high resolution power and sensitivity, has been successfully applied to the determination of fatty acids in vegetable oils (5) and fatty acids and cholesterol in biological fluids (6). In the latter study, large differences in the physical and chemical properties between fatty acids and sterols presented 2 difficulties for their simultaneous determination. The first was the side reactions that sterols undergo during methylation of the fatty acids by acid, methoxide, or boron trifluoride reagents (e.g., dehydration), such as in the boron trifluoride-methanol method of Morrison and Smith (7), in which cholestadiene is produced from cholesterol. More details on this subject can be obtained elsewhere (8). The second difficulty was the GC analysis itself, where 2 separate runs with 2 different column stationary phases were necessary to determine the fatty acids and the sterols.

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In this study, a method was developed for the simultaneous determination of fatty acids and sterols from the low polarity lipid fraction of orange juice. The Morrison and Smith (7) methylation procedure was modified to avoid losses of sterols and a bonded phase fused silica capillary column of intermediate polarity (OV-17) was used, which allowed high temperature programming. In addition, extraction was carried out in a C-18 disposable minicolumn. The method is simple, rapid, reproducible, and applicable to low levels of analytes.

METHOD

Apparatus

(a) *Gas chromatograph.*—Hewlett-Packard Model 5880 with split/splitless capillary injection system and flame ionization detector equipped with HP 7671A autosampler (Hewlett-Packard, Avondale, PA 19311).

(b) *GC column.*—J&W bonded phase fused silica DB 1701, 0.25 mm id × 30 mm (Alltech Associates, Inc., Deerfield, IL 60015).

(c) *Centrifuge.*—International Clinical Centrifuge, bench top (The Chemical Rubber Co., Cleveland, OH 44114).

(d) *Vacuum extraction system.*—Model "Baker" 10 SPE with vacuum regulator, using extraction columns (Baker 6 mL disposable columns and 10 SPE octadecyl (C-18) with 1 g packing material (J. T. Baker Chemical Co., Phillipsburg, NJ 08865).

(e) *Heating module.*—Pierce Model 18800 Reacti-Therm (Pierce Chemical Co., Rockford, IL 61105).

(f) *Evaporating unit.*—Pierce Model 18780 Reacti-Vap.

(g) *Reaction vials.*—Wheaton Micro Product V, with open-top screw cap, cone-shape bottom, and Teflon-face silicone septa (Morgan Scientific Corp., North Strong Division, Rockville, MD 20850).

(h) *Septum vials.*—Pierce 7 mL open-top screw cap, with Teflon-face silicone septa.

(i) *Automatic pipet and plastic tips.*—Oxford continuously adjustable sampler micro pipet (200–1000 μL) and tips (Fisher Scientific Co., Pittsburgh, PA 15219).

(j) *Combined GC/mass spectrometric (MS) data system.*—Finnigan Model 3300F (Finnigan MAT, San Jose, CA 94086).

(k) *Fourier transform-infrared (FT-IR) spectrophotometer.*—Digilab Model FTS-10 (Digilab, Cambridge, MA 02139).

(l) *Emission spectrometer.*—Jarrell Ash Model 975 Plasma Atomcomp with dedicated minicomputer (Jarrell Ash, Division of Allied Analytical Systems, Waltham, MA 02254).

(m) *Ultrasonic bath.*—Bransonic 221 (Branson Cleaning Equipment Co., Shelton, CT).

Reagents

(a) *Solvents.*—Isooctane, hexane, methanol, isopropanol, ethylene chloride, and benzene, LC grade (Burdick & Jackson Laboratories Inc., Muskegon, MI 49442).

(b) *Tris-(hydroxymethyl) aminomethane (Ultrol).*—(Calbiochem-Behring Corp., La Jolla, CA 92037).

Table 1. Precision of automatic injection for fatty acid methyl esters and sterol silyl derivatives in orange juice[a]

Compound	Mean, mg/L[b]	CV, %
Myristic acid	4.03	1
Palmitoleic acid	9.20	1
Palmitic acid	36.20	1
Oleic acid	16.00	1
Linoleic acid	101.00	1
Stearic acid	5.20	2
Linolenic acid	19.30	1
Erucic (Istd) acid	10.00	1
Cholesterol	1.80	3
Campesterol	9.63	3
Stigmasterol	5.51	3
β-Sitosterol	72.00	3
Δ7-Stigmasterol	3.90	4

[a]California navel orange frozen concentrate.
[b]Mean of 9 injections from the same vial.

(c) *Tris buffer.*—Transfer ca 12.1 g Tris to 500 mL beaker and dissolve in ca 100 mL water. Dilute to ca 450 mL with water. Adjust pH to 8.6 with 6N HCl with stirring. Transfer to 500 mL volumetric flask and dilute to volume with water.

(d) *Fatty acids and fatty acid methyl esters.*—Myristic, palmitoleic, palmitic, oleic, linoleic, stearic, linolenic, and erucic acids and their respective methyl esters in individual containers; 99% pure (Applied Science Laboratories, State College, PA 16801).

(e) *Sterols.*—Campesterol, cholesterol, stigmasterol, and β-sitosterol; 98% pure (Alltech Associates).

(f) *Triglycerides.*—Tripalmitin, triolein, trilinolein, and tristearin; 99% pure (Nu-Chek-Prep, Inc., Elysian, MN 56028).

(g) *Silylating reagent.*—Sil-Prep 1 mL ampules (Alltech Associates).

(h) *Methylating reagent.*—BF_3–15% methanol (Alltech Associates).

(i) *Morrison's reagent.*—35% methylating reagent–30% benzene–35% methanol.

(j) *Phospholipids.*—Phosphatidylethanolamine (PE), phosphatidic acid (PA), and phosphatidylcholine (PC); 99% pure (Supelco Inc., Bellefonte, PA 16823).

(k) *3,5-Di-tert-butyl-4-hydroxytoluene (BHT).*—99% pure (Aldrich Chemical Co., Milwaukee, WI 53233).

(l) *Hydrocarbons.*—C15, C23, and C34; 99% pure (Alltech Associates).

(m) *Standard solutions.*—*Solution 1:* Transfer 25 mg of each fatty acid methyl ester (see *Reagents* (d)) to 25 mL volumetric flask and dilute to volume with methylene chloride (1 mg/mL). *Solution 2:* Pipet 250 μL solution 1 into 25 mL volumetric flask and dilute to volume with internal standard solution 9 (fatty acids, 10 μg/mL; hydrocarbons, 8 μg/mL). *Solution 3:* Prepare as standard solution 1, using the fatty acids instead of the methyl esters. *Solution 4:* Transfer 40 mg of each sterol (cholesterol, campesterol, stigmasterol, and β-sitosterol) to 20 mL volumetric flask and dilute to volume with methylene chloride (2 mg/mL). *Solution 5:* Take 80 μL solution 4, evaporate solvent, add 100 μL Sil-Prep, and treat as in procedure below, dissolving silylated extracts in 4 mL internal standard solution 9 (sterols, 20 μg/mL; hydrocarbons, 8 μg/mL). Keep at −4°C for ≤1 week. *Solution 6:* Transfer 200 mg of each triglyceride (tripalmitin, triolein, trilinolein, and tristearin) to 200 mL volumetric flask and dilute to volume with methylene chloride (1 mg/mL). *Solution 7:* Transfer 25 mg of each phospholipid (PE, PA, and PC) to 25 mL volumetric flask and dilute to volume with methylene chloride (1 mg/mL).

(n) *Internal standard solutions.*—*Solution 8:* Transfer 200 mg of each hydrocarbon (C15, C23, and C34) to 200 mL volumetric flask and dilute to volume with isooctane (1 mg/mL). *Solution 9:* Pipet 2 mL solution 8 into 250 mL volumetric flask and dilute to volume with isooctane (8 μg/mL). *Solution 10:* Transfer 250 mg erucic acid to 250 mL volumetric flask and dilute to volume with isopropanol (1 mg/mL).

(o) *BHT solution.*—1% in isopropanol.

Procedure

Use single-strength orange juice products directly; reconstitute frozen concentrates according to the manufacturer's instructions.

Combine 2 mL orange juice, 40 μL 1% solution of BHT in isopropanol, and 2 mL Tris buffer in 5 mL test tube. Place test tube in ultrasonic bath for 1 min, then in water bath at 37°C for 15 min. Adjust pH to 6–7 with 2.5N HCl (ca 3 drops) (test orange juice portion).

Press (with glass rod) small glass wool plug on top of C-18 packing to filter orange juice solids and to eliminate void space between frit and column packing. Wash C-18 column with two 6 mL portions each of hexane, methylene chloride, methanol, and water, in that order, adding next volume of solvent before level of last solvent reaches packing material. Vacuum should be ≤1 in. Hg (25 mm Hg) or resulting flow ≤ 1 mL/min. Add test orange juice portion with automatic pipet before second 6 mL portion of water reaches packing level. Add 40 μL internal standard solution 10 to column. Rinse pipet tip and test tube with 5 mL water and pour washings

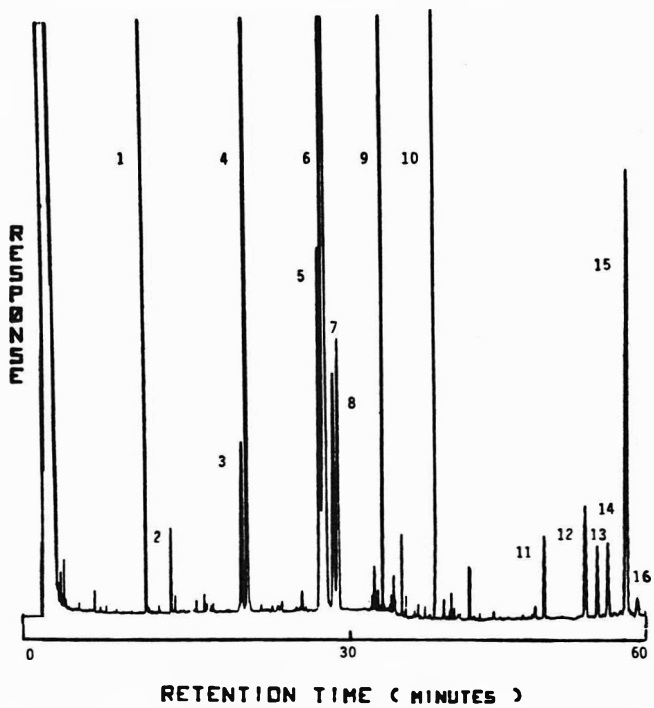

Figure 1. Gas chromatogram (column-compensated) of orange juice extract (California navel).

Operating conditions: temperatures—detector 300°C, injector (splitless) 250°C, column oven, programmed at 80°C for 2 min, to 190°C at 10°/min, to 208°C at 1°/min, to 272°C at 30°/min; gas flow rates—helium carrier gas 1 mL/min at 11 psi, helium make-up gas 100 mL/min at 30 psi, detector gases, air 375 mL/min at 30 psi, hydrogen 30 mL/min at 20 psi; analysis time 65 min; purge delay 1 min; chart speed 0.25 cm/min. Peaks: 1, C15 hydrocarbon; 2, methyl myristate; 3, methyl palmitoleate; 4, methyl palmitate; 5, methyl oleate; 6, methyl linoleate; 7, methyl stearate; 8, methyl linolenate; 9, C23 hydrocarbon; 10, methyl erucate (Istd); 11, cholesterol; 12, campesterol; 13, stigmasterol; 14, β-sitosterol; 15, C34 hydrocarbon; and 16, Δ7-stigmasterol. One μL injection volume corresponds to 0.5 μL orange juice.

Table 2. Recovery of fatty acids and sterols from the C-18 column step added at levels of 10 and 40 mg/L, respectively, to orange juice

Peak[a]	Rel. RT[b]	Compound	Rec.,%[c]	CV, %
2	1.943	myristic acid	110	10
3	1.533	palmitoleic acid	85	6
4	1.517	palmitic acid	90	4
5	1.161	oleic acid	83	3
6	1.151	linoleic acid	96	4
7	1.136	stearic acid	90	4
8	1.122	linolenic acid	90	4
10	0.881	erucic (Istd) acid	95	4
11	1.133	cholesterol	96	3
12	1.038	campesterol	98	3
13	1.013	stigmasterol	98	3
14	0.959	β-sitosterol	113	4
16	0.938	Δ7-stigmasterol	100	4

[a]See Figure 1.
[b]For fatty acids, relative retention time (RT) = RT C23 hydrocarbon/RT fatty acid; for sterols, relative RT = RT C34 hydrocarbon/RT sterol.
[c]Mean of 5 replicates.

into column before it dries. Drain column 5 min; then place in centrifuge tube holder containing a piece of absorbent paper in the bottom to absorb water, and centrifuge at ca 1500 rpm for 15 min. Elute lipids from column with 4 mL methylene chloride and collect eluate in reaction vials placed inside "Baker" 10 system (cardboard or Styrofoam rack may be improvised to keep vials in place). Evaporate to dryness under nitrogen at 40°C. Add 1 mL Morrison's reagent. Cap vials tightly, and place in heating block at 85°C for 45 min. Let vials cool, add 2 mL 25% saturated NaCl solution, shake, and extract with two 1 mL portions of methylene chloride. Transfer each extract to 7 mL septum vial with automatic pipet. Evaporate extract to dryness at 40°C under nitrogen. Add 200 μL (ca 15 drops) Sil-Prep to vial, cap, heat at 40°C 15 min, and evaporate to dryness as above. Finally, add 4 mL internal standard solution 9, sonicate vial 2 min, and then centrifuge at ca 1500 rpm for 5 min. Transfer 1 mL solution to automatic sampler vial.

Gas Chromatography

Set autosampler injection volume at 1 μL. Wash fused silica column with methylene chloride every 6 months to remove impurities.

Quantitate fatty acids present in orange juice, using internal standard method. Formula for internal standard (Istd) calculation is:

$$\text{mg/L } y = \text{amt ratio} \times \text{amt Istd} \times \text{multiplier}$$

where y = compound of interest and amt ratio = (area y/area Istd) × (response y/response Istd).

Obtain response (amt ratio) from calibration of the gas chromatograph using standard solution 2 (fatty acid methyl esters, 10 μg/mL; hydrocarbons, 8 μg/mL). The multiplier is 2 (dilution factor) and results are expressed in mg/L. Amt Istd corresponds to amount internal standard in 1 μL injection of sample (10 ng).

Due to lack of a suitable internal standard for sterols, they were quantitated by the external standard method, using standard solution 5 (silylated sterols) for calibration. Formula for calculation is:

$$\text{mg/L } y = \text{area } y \times \text{response } y \times \text{multiplier}$$

The multiplier is also 2 in this case.

Standard solution 2 contained hydrocarbons (besides the fatty acid methyl esters) that served as reference standards to locate the other peaks if there was any change in retention time (RT). An RT window of 5% was used for the reference peaks, while the other peaks had an RT window of 0.5%.

Method Evaluation

Recovery Studies

Recovery of fatty acids and sterols.—Five 2 mL aliquots of orange juice were fortified with 40 μL standard solution 3 and 80 μL standard solution 4 just after the sample was poured into the C-18 column. Five other nonspiked aliquots were also analyzed.

Calculations were as follows:

$$\text{Rec., \%} = (\text{mean mg/L spiked} - \text{mean mg/L nonspiked}) \times 100/\text{mg/L added}$$

where mg/L added = 10 for fatty acids and 40 for sterols.

Recovery of triglycerides.—Standard solution 6 (40 μL) was added directly to a C-18 column containing 2 mL Tris buffer and 2 mL water at pH ca 6 and analyzed according to the *Procedure*. Recoveries were determined on the basis of experimental yield of fatty acids compared to the theoretical yield.

Recovery of phospholipids.—To determine if phospholipids coeluted with the low polarity fraction of the lipids, 40 μL standard solution 7 was added to a C-18 column and analyzed as described above for the triglycerides. The recoveries were also calculated as for the triglycerides.

Inductively Coupled Plasma (ICP) Emission Spectrometry for Phosphorus

Two mL orange juice was added to 2 mL Tris buffer in a test tube and placed in a water bath at 37°C for 15 min. The

Table 3. Fatty acid and sterol content (mg/L) of 10 orange juice samples[a]

		Valencia		Hamlin		Pineapple		Temple	
Peak[b]	Compound	Mean	SEM[c]	Mean	SEM	Mean	SEM	Mean	SEM
2	myristic acid	1.3	0.1	1.0	0.1	1.3	0.3	1.6	0.1
3	palmitoleic acid	4.6	0.2	3.8	0.3	3.8	0.2	5.6	0.1
4	palmitic acid	17.5	0.2	23.8	5.7	19.6	1.8	25.0	1.6
5	oleic acid	9.7	0.9	8.4	1.6	8.3	1.5	16.4	1.1
6	linoleic acid	64.0	3.8	70.0	5.5	68.0	5.5	83.0	2.9
7	stearic acid	4.4	0.1	4.7	0.1	5.9	2.6	5.3	0.6
8	linolenic acid	13.6	1.3	19.3	3.5	15.4	3.3	30.0	0.6
11	cholesterol	1.0	1.2	0.7	0.2	1.8	1.4	3.0	0.1
12	campesterol	27.0	1.0	20.0	1.3	24.7	4.1	25.0	1.1
13	stigmasterol	5.2	0.2	3.1	0.1	4.9	1.3	4.8	0.2
14	β-sitosterol	60.0	1.7	57.0	5.7	68.0	8.5	61.0	3.2
15	Δ7-stigmasterol	3.4	0.2	4.2	0.7	3.9	0.9	6.4	0.3

[a]Three samples each of Temple and Valencia and 2 samples each of Pineapple and Hamlin.
[b]See Figure 1.
[c]Standard error of the mean. These values are not statistical errors; they only show the fluctuation in the amounts of the compounds among 10 different orange juice products.

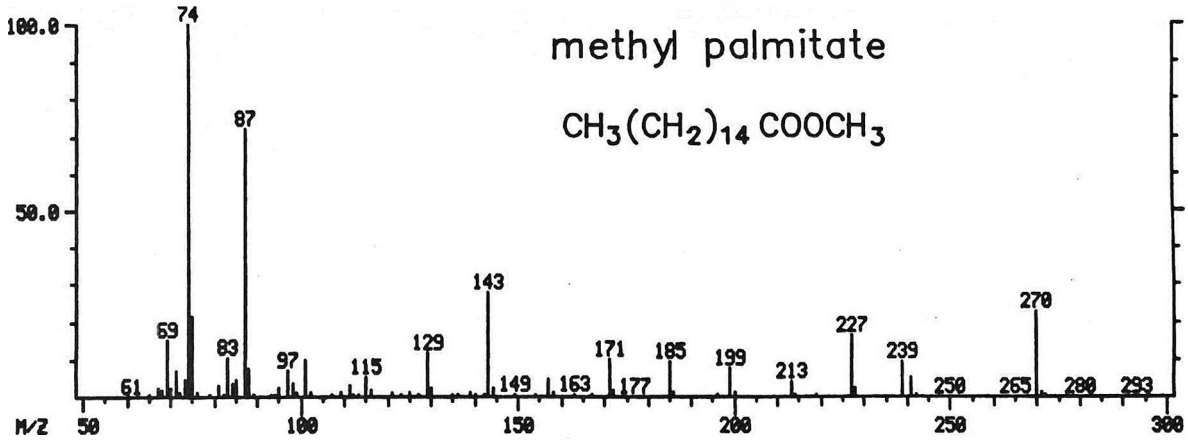

Figure 2. GC/MS spectrum of methyl palmitate (peak 4 in Figure 1).

pH was adjusted to 6 and the sample was passed through the C-18 column as described for the procedure. The aqueous eluate from the column was diluted to 10 mL with water and submitted for ICP spectrometric determination of total phosphorus together with a similarly prepared orange juice sample without column chromatography. Samples and blanks were wet-ashed (9) and analyzed.

Fourier Transform Infrared Spectroscopy

Approximately 0.5 g of each sterol (campesterol, β-sitosterol, cholesterol, and stigmasterol) was silylated with 1 mL Sil-Prep. After evaporation of the pyridine at 40°C in a water bath, under nitrogen, 1 mg of each compound was analyzed in a KBr disk in the 400–4000 cm^{-1} range with a resolution of 4 cm^{-1}. Nonsilylated sterols were similarly determined. The purity of the silyl ethers thus prepared was determined by comparing the spectrum of the product with the spectrum of the starting material using the Digilab least squares curve-fitting computer program (10).

Gas Chromatography/Mass Spectrometry

A 12 m SE-54 fused silica capillary column was used for GC/MS analysis of the extracts. The end of the column was inserted through the transfer line into the mass spectrometer until it was at the entrance to the ion source. Helium carrier gas pressure and flow controls were adjusted to achieve a linear velocity of 27 cm/s through the column at ambient temperature. This produced a vacuum manifold pressure of 10^{-5} torr. The injection port and transfer line temperatures were adjusted to 230°C. Column temperature was 50°C during splitless injection of sample. One min after injection, the column was heated to 270°C at 20°/min. During data acquisition, the mass spectrometer was repetitively scanned from 60 to 660 daltons every 1.6 s under data system control.

Results and Discussion

Simultaneous determination of fatty acids and sterols presented 3 major difficulties: *(1)* discrimination due to differences in volatilities between sterols and fatty acids, *(2)* resolution of the 2 chemically different classes of compounds on a single column, and *(3)* degradation of sterols while methylating the fatty acids.

Discrimination problems were obvious in our system, especially when manual splitless injection was used. Yang et al. (11) studied the variables involved in obtaining good results with the splitless capillary injection technique, and established a set of values for optimal performance with minimized discrimination. Their recommended injection rate of 1 μL/s was suitable for our system. A rate lower than 1 μL/s resulted in a decrease in peak areas for the sterols, and a greater rate produced smaller peak areas for the fatty acids. Because of the difficulty in obtaining reproducible injection rates with manual injection, low precision in the peak areas was obtained (20% coefficient of variation (CV)). On the other hand, when automatic injection was employed, this rate was constant and reproducible peak areas were obtained (3% CV) (Table 1). Manual on-column injection also gives high analytical precision (2.8% CV) because all the analyte is deposited in the column (12, 13); however, the disadvantage of on-column injection (in the case of the analysis of many samples) is that it has not yet been automated for most GC systems.

The problem of simultaneous resolution of the fatty acids and sterols was solved by the use of a DB 1701 (OV-17) column. As seen in Figure 1, this column provided satisfactory resolution of these 2 chemically different classes of compounds. Although the *cis*- and *trans*-isomers of the unsaturated fatty acids are not completely resolved, according to Nordby and Nagy (1) the *trans*-isomers constitute a minimal percent of the total fatty acid content of orange juice. Another advantage of the bonded phase column is its stability. No significant changes in its performance were noted after using it for >1 year.

In the simultaneous determination of fatty acids and sterols, there is also the problem of side reactions of the sterols in the presence of methylation agents. When methylation was performed by the Morrison and Smith (7) procedure at 100°C as recommended, other peaks in addition to the sterol peaks were obtained in the same region of the gas chromatogram. At 80°C, however, this did not occur, and the recoveries of the sterols were >90% compared to 60% at 100°C. Methylation of the fatty acids was not affected by lowering the temperature.

After the above problems were solved as described, the method was validated. To carry out the recovery studies, it was necessary to determine, first, if phospholipids, the polar fraction of the orange juice lipids, were extracted quantitatively by the C-18 column, and, second, if any of these compounds coeluted with the low polarity lipids upon elution of the C-18 column with methylene chloride. The first area was investigated by ICP spectrometric determination of total phosphorus in orange juice before and after the C-18 column. The ICP spectrometric results showed that an amount of phosphorus equivalent to 300 mg phosphatidylcholine/L remained in the column. This result agrees with the values

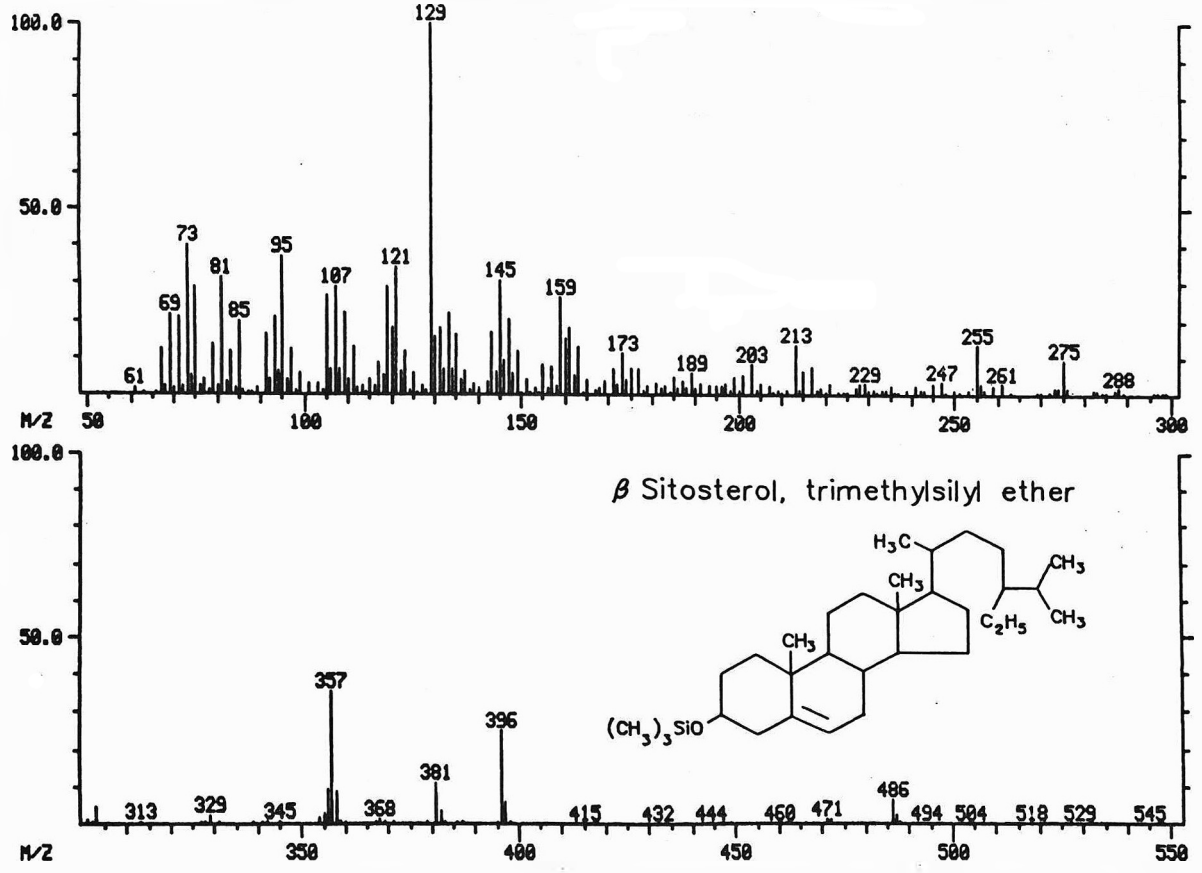

Figure 3. GC/MS spectrum of β-sitosterol trimethylsilyl ether from orange juice extract (peak 15 in Figure 1).

reported by Vandercook et al. (14) for total phospholipids in orange juice. In addition, no fatty acids (or sterols) were obtained when the orange juice was extracted with methylene chloride after the C-18 column extraction. Thus, extraction of the phospholipids by the C-18 column was complete. The second aspect was studied using phospholipid standards in an aqueous matrix. The suspension was passed through the C-18 column and the phospholipids were eluted by methylene chloride. Phospholipids did not start to elute until 11 mL solvent was used. These results showed that the phospholipid fraction did not overlap the low polarity lipid fraction if <10 mL methylene chloride was used.

Recovery studies of the low polarity lipids were then carried out. Standards of each of the fatty acids and sterols naturally found in orange juice were added to orange juice in the column and not at the beginning, that is, in the test tube. It was not possible to obtain reproducible results by spiking in the test tube because of insolubility of standard fatty acids and sterols in the aqueous orange juice matrix. Therefore, the data shown in Table 2 correspond to the recoveries of the procedure from the C-18 column step onward. Standard free fatty acids and sterols were used, despite the fact that the low polarity lipid fraction of orange juice also contains tri-, di-, and monoglycerides and steryl esters. It was not possible to quantitate the glyceryl and steryl esters as such to determine their recoveries because after hydrolysis and derivatization, these compounds were reduced to fatty acid methyl esters and silyl ethers of the sterols. Therefore, the GC step was designed for these derivatives only.

It was important to know that elution of all components of the low polarity lipid fraction from the C-18 column was complete using <10 mL methylene chloride; therefore, indirect determinations of the completeness of the elution were carried out by *(1)* recovering triglycerides from an aqueous matrix as described under Method, *(2)* studying the fractionated elution patterns from the C-18 column, and *(3)* determining the total low polarity lipids. The recovery of triglycerides from the C-18 column was >95%, using 3 mL methylene chloride. Although this experiment was not carried out for the di- and monoglycerides or the steryl esters, on the basis of their chemical structure and solubility in methylene chloride, it was postulated that the steryl esters eluted first and di- and monoglycerides last. Studies of the elution pattern of the fractions showed that no lipids were eluted between 4 and 11 mL methylene chloride, after which the phospholipids started to elute. About 350 mg total low polarity lipids/L were found, in agreement with Nagy and Nordby (2), who reported 400 mg total low polarity lipids/L orange juice. Based on the 3 studies described, 5 mL methylene chloride was sufficient to completely elute the low polarity lipids from the C-18 column.

The external standard method was used for quantitation in the recovery studies (Table 2) for both fatty acids and sterols. The fatty acids in the orange juices were quantitated using the internal standard method, with erucic acid as the internal standard. A suitable internal standard for sterols was not found. Cholesterol would have been a good standard, but it was found to be present in all the orange juices analyzed (Table 3). Therefore, the external standard method was used for quantitation of the sterols.

The results in Table 3 show some indication of the differences in content of fatty acids and sterols according to cultivar. However, more data and further statistical treatment of these data are needed to confirm these findings. Other studies will be carried out on this subject area.

Method validation also included blank runs, which showed no contaminating compounds that interfered with the GC peaks for the fatty acid methyl esters and sterol silyl ethers.

FT-IR spectroscopy was used to determine the purity of the silylated sterol standards. The reaction was complete using 200 μL silylating reagent, but the standards decomposed rapidly after 1 day. However, if they were kept in a freezer, the silyl derivatives could be used for 1 week.

Confirmation of identity by GC/MS was performed for all the numbered fatty acids and sterols in Figure 1. GC/MS spectra for 2 of these peaks are shown in Figures 2 and 3. Cholesterol and Δ7-stigmasterol were the only compounds in orange juice not reported previously in the literature. The other compounds have been reported and studied before (1, 2).

In conclusion, the results presented in this paper show that fused silica capillary GC can be used for simultaneous quantitative determination of fatty acids and sterols in orange juice. Also, use of the C-18 extraction minicolumns considerably reduced the time of analysis and the volumes of solvents required. The procedure is simple, rapid, and reproducible. In addition, it is potentially applicable to other fruit juices.

Acknowledgments

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TECHNICAL COMMUNICATIONS

Chemical Derivatization Analysis of Pesticide Residues. X. Analysis of Ten Acid Herbicides in Natural Waters[1]

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An improved and augmented gas chromatographic (GC) method using a capillary column and electron capture detector was developed for determination of 10 common acid herbicides in natural water. The herbicides were extracted with methylene chloride after the water sample was acidified to pH < 1. Concentrated extracts in acetone were derivatized with pentafluorobenzyl bromide (PFBBr) to form the corresponding PFB esters. Derivatives were cleaned up on a deactivated silica gel column. A SE-54 fused silica capillary column was used to separate and identify the products. Using this procedure, the method was successfully validated for herbicide concentrations as low as 0.05 μg/L in natural waters. Recoveries of water samples fortified with the 10 herbicides ranging from 1.0 to 0.05 μg/L were 73 to 108% with the exception of picloram which was only 59% recovered at 0.1 μg/L.

The use of phenoxy acid herbicides to control the growth of broad-leaf weeds has been widespread in the western provinces of Canada. One report (1) estimated sales of 4.5×10^6 kg (acid equivalent) 2,4-D and up to 10^5 kg other phenoxy acid herbicides in 1975 and 1976. Indeed, residues of these herbicides were found in many water samples collected in the prairie provinces of Canada during the 1970s (2). The interest in and concern over existence of phenoxy residues in the environment are undoubtedly related to the toxicity, persistence, and known and suspected carcinogenicity of the parent herbicides and metabolites, as well as to the presence of the extremely toxic TCDDs as side products in the 2,4,5-T and silvex formulations.

The chemistry, analysis, and environmental impact of acid herbicides have recently been reviewed by Que Hee and Sutherland (3) and by Sirons, Chau, and Smith (4). Among the various approaches, phenoxy residues are usually determined as their methyl (5, 6), 2-chloroethyl (5, 7, 8), 2,2,2-trichloroethyl (8, 9), or pentafluorobenzyl (PFB) esters (5, 10–12). Methyl derivatives of phenoxy acids were the choice of many workers because they were formed with few interfering side products and provided good sensitivity for herbicides with 2 or more chlorines per molecule. However, for monochlorinated herbicides such as MCPA and MCPB, the electron capture detector sensitivities to the methyl or the 2-chloroethyl esters were very low (5, 13). To meet the objectives of our Water Quality Laboratory which required a multiresidue method of low detection limit for the 10 commonly used acid herbicides, namely, dicamba, MCPA, 2,4-DP, 2,3,6-TBA, 2,4-D, silvex, 2,4,5-T, MCPB, 2,4-DB, and picloram, we opted for the stable and sensitive PFB ester derivatives.

In this paper, we report an augmented, multiresidue method for determining the above 10 acid herbicides in natural waters by solvent extraction and formation of PFB esters. The derivatives are quantitated by capillary column gas chromatography with electron capture detection. This method has a detection limit as low as 0.05 μg/L for 1 L water.

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[1]For part IX of this series, see H.-B. Lee, L. D. Weng, & A. S. Y. Chau (1984) *J. Assoc. Off. Anal. Chem.* **67**, 1086–1091.

Experimental

Apparatus

(a) *Gas chromatograph.*—Hewlett-Packard Model 5880A equipped with ^{63}Ni electron capture detector, Model 7671A autosampler, level 4 terminal, and split-splitless capillary column injection port. Operating temperatures: injection port 250°C, detector 300°C, column, see below. Splitless valve on for 30 s. Detector make-up gas, argon–methane (95 + 5), 25 mL/min; helium carrier gas flow rate, see below. Inject 2 μL sample.

(b) *GC columns.*—*(1)* 30 m × 0.25 mm id SE-54 (J & W Scientific, Inc.) or 30 m × 0.25 mm id DB-5 (J & W Scientific Inc.) fused silica capillary column. Temperature program: initial, 70°C, hold 0.5 min, programming rate 1, 30°/min (70°–200°C), hold at 200°C for 10 min, programming rate 2, 30°/min (200°–220°C), hold at 220°C for 15 min, column head pressure 20 psi. *(2)* 12 m × 0.2 mm id OV-1 (Hewlett-Packard Part No. 19091-60312) fused silica capillary column. Temperature program: initial, 70°C, hold 0.5 min, programming rate 1, 30°/min (70°–160°C), programming rate 2, 2°/min (160°–200°C), hold at 200°C for 10 min, column head pressure 10 psi. *(3)* 12 m × 0.2 mm id Carbowax 20M (Hewlett-Packard Part No. 19091-60010) fused silica capillary column. Temperature program: initial 70°C, hold 0.5 min, programming rate 1, 25°/min, (70°–140°C), programming rate 2, 1°/min (140°–175°C), programming rate 3, 5°/min (175°–200°C), hold at 200°C for 15 min, column head pressure 10 psi.

Reagents

Use distilled-in-glass or pesticide grade solvents.

(a) *Herbicides.*—Analytical grade standards obtained from manufacturers or Environmental Protection Agency (HERL, Research Triangle Park, NC 27711) and used without further purification. Dissolve 100 mg individual herbicide in 100 mL ethyl acetate.

(b) *Spiking solution.*—Pipet appropriate amounts of each herbicide stock solution into 100 mL volumetric flask and dilute with acetone to generate acid herbicide mixture containing 10 μg/mL for each herbicide except for 20 μg/mL of 2,4,5-T, MCPB, 2,4-DB, and picloram. Use 100 μL of this mixture to spike water samples at highest fortification level.

Table 1. Retention times (min) of 10 acid herbicide-PFB esters on different capillary columns

Parent Herbicide	12 m × 0.2 mm Carbowax 20M	30 m × 0.25 mm SE-54	12 m × 0.2 mm OV-1
Dicamba	30.11	13.02	14.78
MCPA	36.88	13.32	14.95
2,4-DP	32.68	13.85	15.56
2,3,6-TBA	33.14	14.09	15.74
2,4-D	43.88	15.51	17.12
Silvex	41.11	17.35	19.97
2,4,5-T	45.90	19.01	21.81
MCPB	50.10	19.85	23.17
2,4-DB	51.48	22.15	25.43
Picloram	—	26.71	28.92

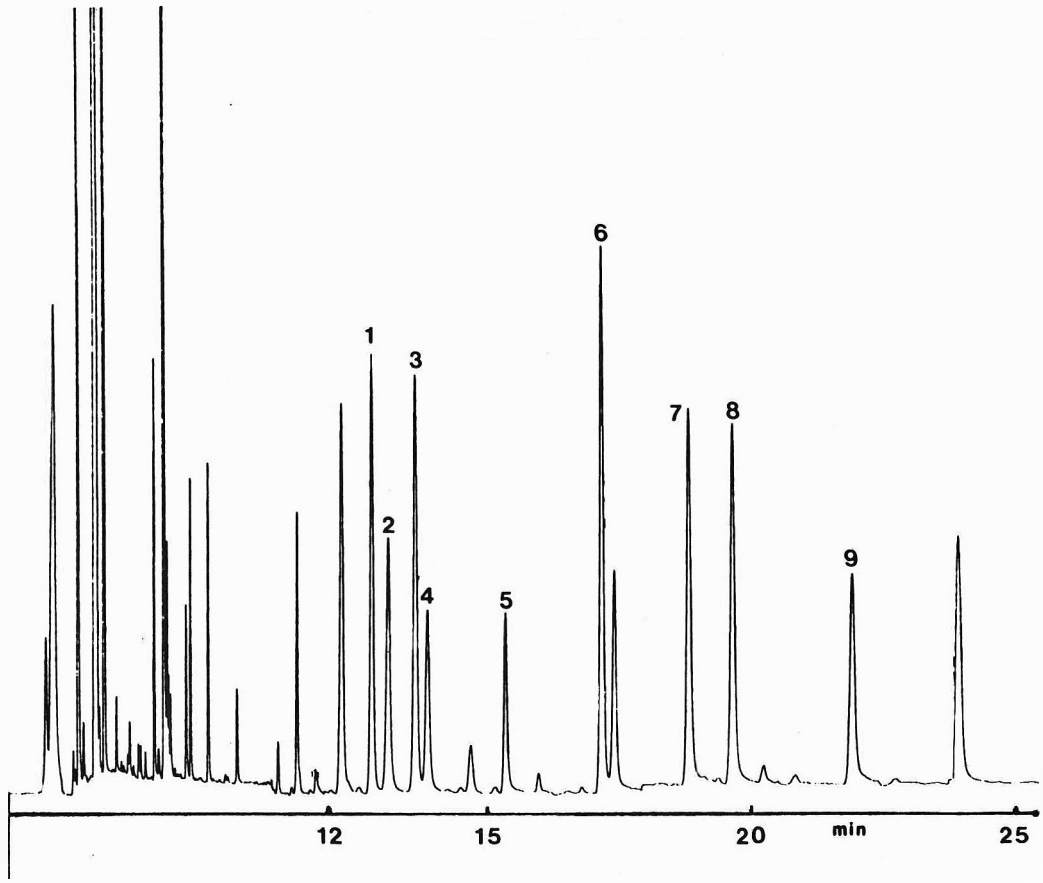

Figure 1. Gas chromatogram of standard mixture of 9 acid herbicide PFB esters as chromatographed on 30 m SE-54 silica capillary column. Approximately 100–200 pg of each component was injected. (1) dicamba; (2) MCPA; (3) 2,4-DP; (4) 2,3,6-TBA; (5) 2,4-D; (6) silvex; (7) 2,4,5-T; (8) MCPB; (9) 2,4-DB. Note that picloram PFB ester is not shown because it elutes in a different fraction.

Table 2. Mean % recovery of acid herbicides from 1 L pH < 1 fortified pure water samples (averages of 5 or 6 replicates in each case)

	Level of fortification μg/L (SD)		
Herbicide	1.0	0.1	0.01
Dicamba	88.0 (4.1)	95.2 (11.1)	93.2 (2.4)
MCPA	102.4 (9.6)	97.3 (3.5)	97.1 (4.1)
2,4-DP	106.7 (2.5)	104.5 (5.6)	100.4 (7.7)
2,3,6-TBA	85.7 (7.8)	90.0 (7.1)	82.5 (5.1)
2,4-D	94.9 (11.4)	96.0 (6.9)	73.4 (6.3)
Silvex	103.6 (5.1)	93.5 (7.8)	93.5 (2.4)
2,4,5-T[a]	99.0 (6.9)	102.8 (9.8)	78.0 (5.1)
MCPB[a]	98.0 (4.7)	102.7 (4.6)	90.3 (4.5)
2,4-DB[a]	103.3 (7.9)	101.8 (10.8)	66.0 (5.8)
Picloram[a]	69.5 (6.2)	71.3 (6.8)	75.5 (4.8)

[a]These 4 herbicides are spiked twice as high as the other 6 herbicides.

Table 3. Mean % recovery of acid herbicides from 1 L of pH < 1 fortified Lake Ontario water samples

	Level of fortification, μg/L (SD)[a]		
Herbicide	1.0	0.1	0.05
Dicamba	90.0 (5.0)	84.4 (5.1)	90.5 (6.7)
MCPA	90.8 (8.9)	90.9 (9.9)	80.3 (15.1)
2,4-DP	93.9 (2.8)	99.2 (5.0)	97.6 (19.8)
2,3,6-TBA	90.5 (4.9)	88.0 (7.6)	95.5 (17.1)
2,4-D	84.3 (9.8)	108.4 (12.7)	100.7 (22.1)
Silvex	100.4 (5.9)	101.2 (7.1)	88.3 (13.0)
2,4,5-T[b]	91.5 (8.5)	91.3 (7.7)	76.7 (18.0)
MCPB[b]	102.9 (6.9)	96.6 (4.5)	90.3 (10.7)
2,4-DB[b]	101.1 (2.0)	100.1 (5.1)	73.2 (8.1)
Picloram[b]	85.8 (4.5)	59.1 (3.9)	66.4 (11.2)

[a]n = 6.
[b]These 4 herbicides are spiked twice as high as the other 6 herbicides.

For other spiking levels, use 100 μL of an appropriate dilution of this mixture.

(c) *PFBBr reagent.*—See ref. 12.

(d) *K_2CO_3 solution.*—See ref. 12.

(e) *Silica gel.*—See ref. 12.

Fortification of Water Samples

Spike 1 L water with 100 μL acid herbicide mixture in acetone at appropriate concentrations. Stir and equilibrate 30 min before extraction.

Extraction

Stir water sample (1 L), collected in 1.15 L long-neck whiskey bottle or other suitable glass container, using Teflon-coated stirring bar so that vortex formed almost reaches bottom of bottle. Carefully add dilute H_2SO_4 (1 + 1) until pH is ≤ 1 (pH paper).

Extract water sample 3 times using 50 mL aliquots of CH_2Cl_2 as described in ref. 14. Discard water sample after last extraction. Evaporate combined organic extracts at 40°C on rotary evaporator under reduced pressure until volume is ca 20 mL. Add 50 mL benzene to extract and repeat evaporation until just dry to remove remaining CH_2Cl_2 and traces of water in extract. Redissolve residue in four 2 mL portions of acetone and transfer to 15 mL centrifuge tube; mix well.

Esterification

Transfer aliquot of acetone solution to another test tube and esterify herbicides as described in ref. 12. If entire sample is used, concentrate extract to 4 mL before derivatization.

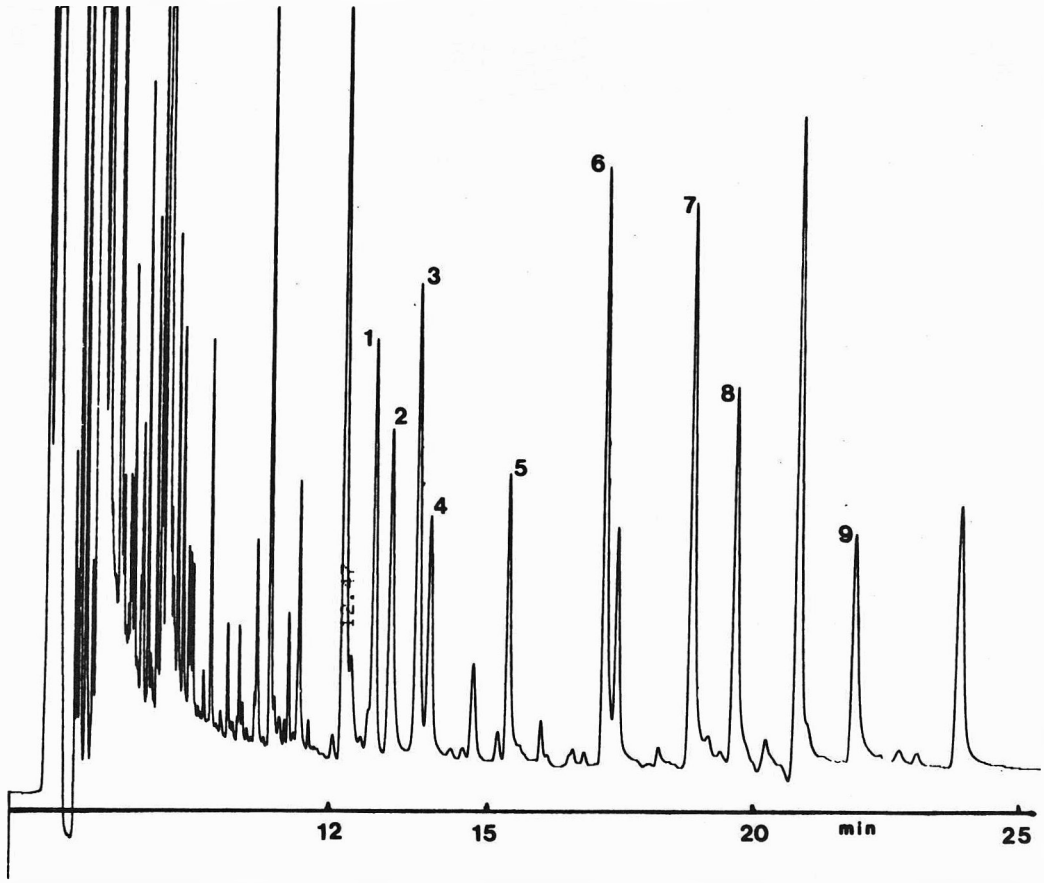

Figure 2. Gas chromatogram of extract derived from Lake Ontario water sample purified at 0.1 μg/L. See Figure 1 for peak identification.

Column Cleanup

Cleanup herbicide PFB esters as described in ref. 12 and replace benzene with toluene in eluants used.

Standard Preparation

Using procedure described above, derivatize and cleanup known amounts of herbicides and use derivatives as standards to quantitate sample extracts.

Results and Discussion

GC Resolution of Acid Herbicide PFB Esters

In a previous paper (12), we reported the GC separation of the 10 acid herbicide PFB esters on 4 different packed columns. Only the Ultrabond-20M column was capable of resolving all 10 components. Studies on sediment samples indicated that satisfactory results could be obtained with this column for herbicide levels of 25 μg/kg or higher (12). Occasionally, a second column of different polarity was required for confirmation and quantitation because of the interference of coextractives. At lower herbicide levels, such as in the case of water samples, it is necessary to use a column of higher efficiency for more reliable identification and quantitation of sample extracts.

Several fused silica capillary columns were evaluated for determining the 10 PFB esters. Similar to the Ultrabond-20M packed column, a 12 m Carbowax-20M capillary column was shown to resolve the 9 PFB esters (see *Experimental*) in fraction A. Again the PFB esters of picloram has an extremely long retention time (> 60 min) on this column. Since this column is known to be less thermally stable, together with the fact that this column has to be operated at its upper temperature limit in the analysis of the PFB esters, it was decided that the Carbowax column was not suitable for long-term use.

All 10 esters were also successfully resolved on a 12 m OV-1 as well as a 25 m or 30 m SE-54 fused silica capillary column. Of the OV-1 and SE-54 columns, for which the orders of elution for the acid herbicide PFB esters were the same, the SE-54 column was used in this work because of its superior resolution (Figure 1). For the retention times of the PFB esters, see Table 1.

Extraction Derivatization and Cleanup

See discussions in refs 5 and 12.

Recoveries of Acid Herbicides from Fortified Water Samples

Replicate pure water samples fortified at 3 different concentrations were analyzed for the 10 acid herbicides. The mean % recoveries (see Table 2) varied from 85 to 106.7% for all except the following cases: At a very low (0.01 μg/L) level, recoveries were slightly lower and, in a few cases, recoveries of 66 to 78% were obtained. Picloram generally gave lower (70 to 80%) recoveries at all fortification levels. The 10 herbicides were also successfully recovered from fortified Lake Ontario water samples at 1.0, 0.1, and 0.05 μg/L (Table 3). However, at the low level of 0.01 μg/L, recoveries of acid herbicides from this Lake Ontario sample were considerably lower and the precision was much worse than those indicated in Table 3. Therefore, a detection limit of 0.05 μg/L rather than 0.01 μg/L was set for the acid herbicides. A typical chromatogram of an extract derived from a Lake Ontario water sample fortified at 0.1 μg/L is shown in Figure 2. Note that in the above discussion as well as in Tables 2 and 3, the levels of 2,4,5-T MCPB, 2,4-DB, and picloram were spiked twice as high as the level indicated.

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On-Line Generation of Cyanogen Chloride in Semiautomated Determination of Niacin and Niacinamide in Food Products

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The current AOAC procedure for semiautomated determination of niacin specifies the use of externally generated cyanogen bromide. Because of the safety concerns in handling this material, we investigated the use of an alternative system of generating cyanogen chloride in situ, using chloramine-T and potassium cyanide. Recovery studies conducted on 9 different food products yielded average recoveries of 101%. A repeatability study resulted in a measured coefficient of variation of 2.9%. The AOAC niacin method was compared with this semiautomated method; 115 paired analyses on 8 different food types over 6 separate analytical replications indicated no significant difference by a paired *t*-test at the 95% confidence level.

The current AOAC procedure (1) specifies the use of externally generated cyanogen bromide for analysis of niacin. The hazards associated with the use of cyanogen bromide are well documented (2–4). This prompted an investigation of alternative reagent systems for niacin determination. One system was the combination of 2 reagents, chloramine-T (3.8% wt/vol.) and potassium cyanide (0.95% wt/vol.) in a mixing coil. This combination generates cyanogen chloride in situ. Cyanogen chloride, a halogen analog of cyanogen bromide, is equally effective in forming the appropriate color with the pyridine-pyrazolone reagent during cyanide analysis (5). Since potassium cyanide reagent can be stored and handled more conveniently and safely than cyanogen bromide, an attempt was made to use the chemistry of the cyanide (5) determination to generate cyanogen chloride in situ and substitute the pyridine portion of the alkaloids for the pyridine-pyrazolone reagent.

METHOD

Apparatus

(a) *Automated analyzer.*—Technicon AutoAnalyzer II system with flow scheme as in Figure 1 (Technicon Instruments Corp., Tarrytown, NY 10591).

(b) *Collection funnels.*—20 mL disposable funnels are convenient.

(c) *Pipets.*—Rapid dispensing pipets are convenient for multiple analyses.

Reagents

(a) *Wetting agent.*—30% aqueous Brij 35 solution (Atlas Chemical Co., Wilmington, DE 19899).

(b) *Phosphate buffer solutions.—(1) Stock solution.*—Dissolve 130 g Na_2HPO_4 and 71 g KH_2PO_4 in ca 900 mL warm water. Cool to room temperature and dilute to 1 L with water *(2)Working solution.*—pH 6.7. Dilute 150 mL stock solution to 1 L with water and add 15 mL wetting agent. Filter through Whatman 2V paper before use. *(3) Sample buffer solution.*—pH 7.6. Dissolve 272 g Na_2HPO_4 and 48 g KH_2PO_4 in ca 1.8 L warm water. Cool to room temperature and dilute to 2 L with water.

(c) *Sulfanilic acid solution.*—10%. Add 100 g sulfanilic acid to ca 500 mL water. Add NH_4OH with mixing until dissolved (ca 40 mL). Adjust to pH 7.0 with HCl (1 + 3) and dilute to 1 L with water. Filter and store in a cool, dark place. Prepare fresh every 2 weeks.

(d) *Chloramine-T solution.*—Weigh 3.85 g chloramine-T into 100 mL volumetric flask containing 70 mL water, and dissolve. Dilute to volume with water. Prepare fresh each day and filter before use. (Caution: Filter solution in hood.)

(e) *Potassium cyanide.*—Weigh 0.95 g KCN into 100 mL volumetric flask containing 70 mL water, and stir to dissolve. Dilute to volume with water. Prepare fresh each day and filter before use. (Caution: Filter solution in hood.)

(f) *Sample wash solution.*—Dilute 3.0 mL wetting agent to 2 L with water and filter through Whatman 2V paper.

(g) *Calcium hydroxide slurry.*—Add 22 g $Ca(OH)_2$ to 200 mL volumetric flask and add ca 100 mL water. Shake to disperse and dilute to volume with water. To use, transfer to 250 mL beaker on magnetic stirrer and stir at rate to ensure homogeneity.

(h) *Basic solution for waste container.*—Dissolve 150 g NaOH in 300 mL water in 4 L reagent bottle. Pump waste into this bottle in hood.

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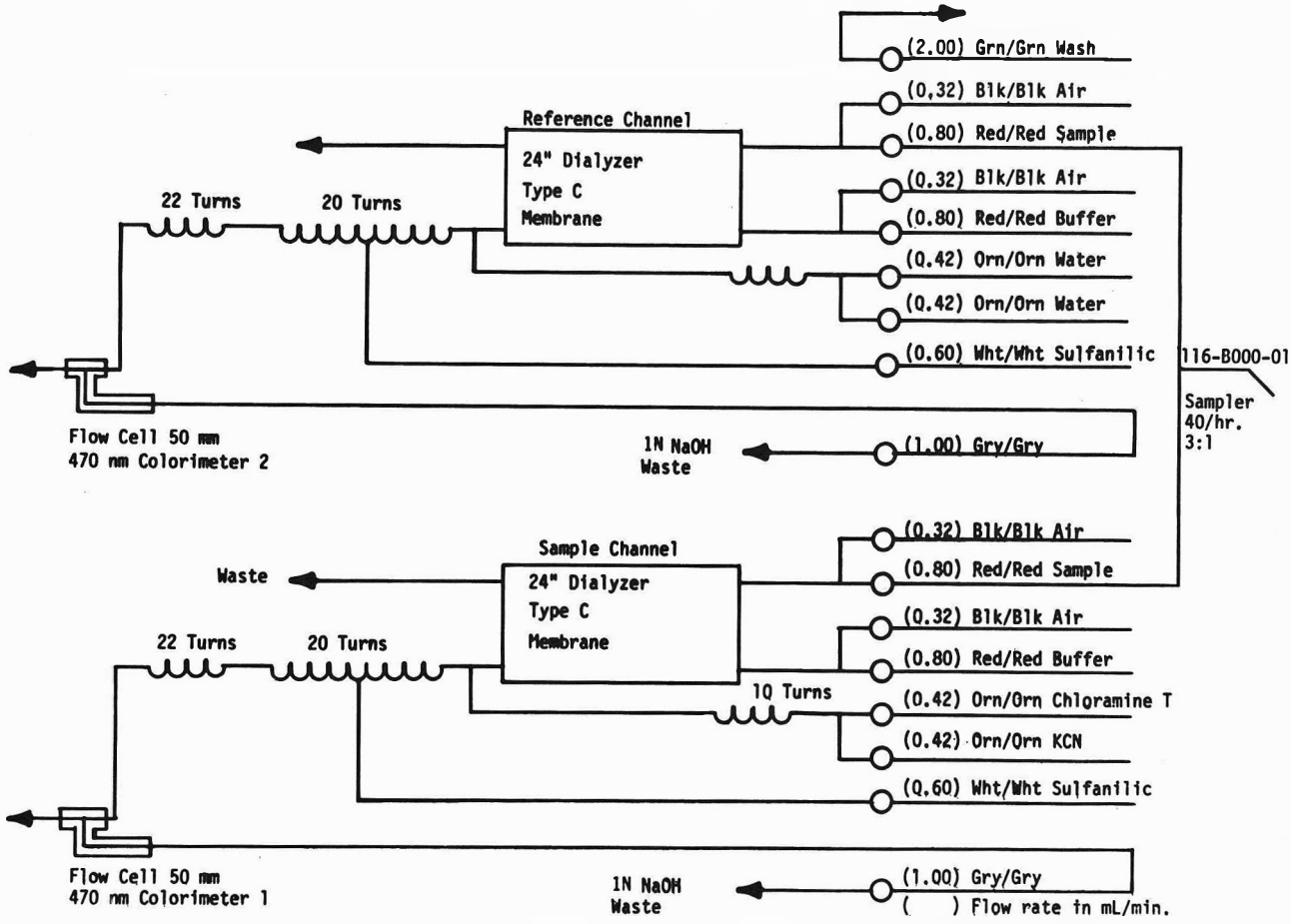

Figure 1. Flow scheme for niacin determination, cyanogen chloride method.

Table 1. Comparison of AOAC semiautomated method with cyanogen chloride method

Food category	No. of samples	Av. niacin, mg/100 g		*t*-Value at 0.05
		Cyanogen chloride	AOAC	
Unfortified vegetables	12	2.34	2.32	0.9774
Enriched wheat flour	28	6.04	6.04	0.903
Rice	18	3.73	3.61	2.4858
Ready-to-eat cereal	29	34.47	34.67	1.0710
Granola bar	4	0.79	0.67	1.658
Cake mix	7	2.34	2.38	0.770
Fortified hot cereal	8	15.38	15.41	0.436
Breaded fish sticks	9	1.17	1.17	0.03
Average	(115)	8.28	8.28	

(i) *Niacin standard solution.—(1) Stock solution.*—10 μg/mL. Weigh 50.0 mg nicotinic acid (stored in desiccator) into 500 mL volumetric flask, dissolve, and dilute to volume with water. Daily, dilute 25.0 mL to 250 mL with water. *(2) Working standard solutions.*—Pipet 5, 4, 3, 2, 1, and 0.5 mL stock solution into 100 mL volumetric flasks containing 5 mL $Ca(OH)_2$ slurry. Add water to adjust final volume to ca 55 mL, and treat standards the same as samples, beginning ". . . autoclave 2 h at 121°C." Final solutions will contain 5.0, 4.0, 3.0, 2.0, 1.0, and 0.5 μg niacin/mL.

Determination

Grind representative portion of sample to pass No. 40 sieve. Accurately transfer weighed portion (1.5 g maximum) of ground sample containing ca 0.2 mg niacin to 100 mL volumetric flask. Add 5 mL $Ca(OH)_2$ slurry, using rapid dispensing pipet. Add ca 50 mL water, cover with foil, swirl, and autoclave 2 h at 121°C. For products that spatter during hydrolysis (high oil content), use 125 mL Erlenmeyer flask and transfer quantitatively to 100 mL volumetric flask after autoclaving.

While solution is still hot, add 10 mL 1.5N HCl (1 + 7) and swirl to dissolve remaining $Ca(OH)_2$. Be sure all $Ca(OH)_2$ is dissolved. Let cool to room temperature (solutions may be stored at this point).

To sample and standard solutions, add 25 mL sample buffer solution (b)*(3)* and 2 drops of wetting agent, and dilute to volume with water. (Precipitate forms and final pH will be ca 6.7.) Shake; filter through Whatman 2V paper (disposable collection funnels are convenient).

Pump high standard solution (5 μg/mL) through system and set recorder pen at 100% with standard calibration adjustment. Aspirate and pump set of standards and sample filtrates through system. Use one standard with every series of 20 samples to correct for any drift. If sample is more concentrated than highest standard, dilute sample solution with working buffer solution (b)*(2)* to bring peak height into range of standards. After all sample filtrates have been run, replace chloramine-T and KCN lines with water. Let pump ca 15 min and resample filtrates to obtain corresponding blank values. Alternatively, dual channel instrument may be used for simultaneous blank corrections as shown in Figure 1.

Calculations

Plot standard curve of A (chart units) of standard minus blank against niacin concentration in μg/mL, drawing line of

Table 2. Precision and accuracy of cyanogen chloride method for determining niacin

	Cyanogen chloride				AOAC
Sample	Av., mg/100 g (±SD)[a]	CV, %	Added, μg	Rec., %[a]	Av., mg/100 g[a]
Canned asparagus	1.13 ± 0.05	4.00	20	101.2	1.00 ± 0.06
Enriched wheat flour	4.35 ± 0.19	4.35	50	96.2	4.30 ± 0.17
Rice	3.87 ± 0.12	2.98	50	98.4	3.83 ± 0.12
Peanut butter crackers	6.91 ± 0.07	1.01	50	106.1	6.96 ± 0.14
Ready-to-eat cereals:					
Honey oat	18.95 ± 0.57	3.02	100	97.1	19.23 ± 0.42
Corn	94.91 ± 1.91	2.01	250	96.2	93.57 ± 2.05
Oat	20.33 ± 1.91	1.18	100	101.9	20.52 ± 2.10
Malt cereal	9.35 ± 0.41	4.40	50	107.6	9.45 ± 0.39
Breaded fish	1.04 ± 0.04	2.92	20	109.9	1.05 ± 0.04
Average	17.87	2.87		101.6	17.77

[a]Average of 3 replicates.

best fit. Read concentration, C, corresponding to A of sample corrected for blank and any shift in baseline during run. For dilution to 100 mL for both samples and standards:

$$\text{mg Niacin or niacinamide/100 g} = C/(10/\text{g sample})$$

where $C = (\mu g/mL) \times 100$.

Results and Discussion

The method using in situ generation of cyanogen chloride was compared to the AOAC automated method; 115 samples from 8 food groups were analyzed by both methods on 6 different dates. Results of a paired t-test indicated no significant difference at the 95% confidence level between the 2 methods (Table 1).

Nine food groups were analyzed in triplicate to test precision of the in situ method. The samples had niacin levels ranging from 1.04 to 94.91 mg/100 g. Coefficients of variation ranged from 1.01 to 4.40%. The overall mean coefficient of variation was 2.87%. Each of the 9 sample groups was spiked with known levels of niacin to test for percent recovery. Recovery ranged from 96.2 to 110%, with an average of 101.6 ± 5.2%. Each of these samples was then analyzed by the current AOAC method. The mean value by the in situ method was 17.87 mg/100 g and by the AOAC method was 17.77 mg/100 g (Table 2).

The results of this study indicate that the modified method gave results equivalent to the current AOAC automated method. The in situ method of generation of cyanogen chloride represents a definite improvement in the safe handling of the reagents used for niacin determination.

References

(1) *Official Methods of Analysis* (1984) 14th Ed., AOAC, Arlington, VA, secs **43.056–43.059**
(2) Harvey, W. R. (1981) *Chem. Eng. News* **5**(59), 49
(3) Grossman, M. I. (1980) *Chem. Eng. News* **35**(58), 43
(4) *Merck Index* (1976) 9th Ed., Merck & Co., Inc., Rahway, NJ, p. 351
(5) *Standard Methods of Analysis* (1971) 13th Ed., American Public Health Association, New York, NY, p. 404

General Referee Report: Forensic Sciences

JOHN W. HICKS
Federal Bureau of Investigation, Laboratory Division, 10th St and Pennsylvania Ave, NW, Washington, DC 20535

Official Methods Review

The General Referee is currently reviewing the chapter on forensic sciences in *Official Methods of Analysis*. This review will focus on the adequacy and appropriateness of existing methods in the context of forensic science practices today.

A review has been conducted to redefine the methods topic areas. Several associate referees for those topics have been appointed and others will be designated in the immediate future.

ASCLD Meeting

The General Referee participated in the annual meeting of the American Society of Crime Laboratory Directors. Numerous discussions were held both in and out of committee meetings on validated methods of analysis for use by crime laboratories.

New Topic Listing and Recommendations

(1) *Grouping tests—blood and other body fluids.*—Henry C. Lee (State Police Forensic Science Laboratory, New Haven, CT). Continue study.

(2) *Screening and confirmatory tests—dried bloodstains.*—Appoint Associate Referee.

(3) *Electrophoretic methods.*—Associate Referee has been appointed (Willard Stuver, Metro-Dade Police Department Crime Laboratory, Miami, FL). Initiate study.

(4) *Enzyme-linked immunosorbent assays for forensic characterization of body fluid stains.*—Appoint Associate Referee.

(5) *Isoelectric focusing methods for forensic characterization of body fluid stains.*—Associate Referee has been appointed (Bruce Budowle, FBI Academy, Quantico, VA). Initiate study.

(6) *Gunshot residues.*—Associate Referee has been appointed (Donald G. Havekost, FBI, Laboratory Division, Washington, DC). Design collaborative study for atomic absorption method.

(7) *Explosives and explosives residues.*—Appoint Associate Referee.

(8) *Soils, geological analysis.*—Associate Referee has been appointed (John Wehrenberg, University of Montana, Missoula, MT). Initiate study.

(9) *Chromatographic methods for forensic characterization of paints and other polymeric materials.*—Appoint Associate Referee.

(10) Discontinue all other previously identified topics.

Received December 12, 1985.

Report of the Archives Committee

CHARLOTTE BRUNNER, *Chairman*
Food and Drug Administration, Division of Drug Chemistry, Washington, DC 20204

Other Members: W. V. Eisenberg; W. Landgraf; R. Pierce; H. Reynolds; E. Sarnoff; H. M. Stahr

The Archives Committee, a newly formed committee, held its first meeting October 30, 1985. The Committee discussed the offer from the Department of Special Collections of Iowa State University to serve as the depository for the AOAC archives. The Department has the proper facilities to preserve and store these materials where they could be available for use by scholars. The Committee recommends to the AOAC Board of Directors that this offer be accepted.

The Committee also discussed categories of materials that should be collected and preserved. Various people were named as possible contacts for oral histories. It was agreed that notices should be placed in the *Referee* and in other publications, such as *Chemical and Engineering News, Analytical Chemistry*, etc., asking people to notify the Committee if they have old AOAC materials that could be preserved and to suggest types of materials that should be added to the collection. Other organizations will be contacted, such as the American Chemical Society, AFDO, the American Oil Chemists' Society, the American Association of Cereal Chemists, land grant universities, the control officials, and trade associations.

Report of the Ways and Means Committee

STANLEY E. KATZ, *Chairman*
Rutgers University, Cook College, New Brunswick, NJ 08903

Other Members: W. P. Cochrane; J. Goleb; M. Malina; L. Perlman; W. Phillips; M. A. Ready

The Ways and Means Committee considered the problems of the static nature of the number of exhibitors at the annual meetings and spring training workshops. The Committee felt that vigorous efforts should be made to attract new exhibitors and that relationships with present exhibitors should be strengthened. To this end, the planning of the annual meetings and spring training workshops should ensure high levels of attendee traffic through the exhibit area.

The question of membership should be resolved as quickly as possible to remove all barriers to membership and end all vestiges of different classes of members. Good analytical science is not a function of the investigator's employer but instead is related to the data produced in collaborative or validation studies. AOAC is no longer an arm of regulatory agencies and hence should not reflect out-dated concepts of membership. An interest in quality analytical methodology should be the criterion of participation. Approval of methodology should not be governed by regulatory requirements and should reflect only the best available methodology. Pragmatically, if significant support is expected from the private sector, different categories of membership cannot be justified.

Increased costs to members should be carefully reviewed so as not to cause erosion of members. Significant increases in membership dues without any tangible return to the member could prove harmful. Other scientific organizations invariably include a journal as part of the fees or the opportunity to purchase journals at significantly reduced rates as part of membership privileges. This model offers some potential advantages for raising revenue from advertising because of significantly larger subscription numbers. By including the *Journal of the AOAC* as part of the membership fee, intangibles such as greater reading of the *Journal* by the membership and the potential for greater participation in AOAC business can be expected.

An expansion in the number of corporate or sustaining members and increased revenues from such memberships are necessary in the future. However, increases in the recommended level of contributions should be such as to minimize or negate an erosion of the number of such members. Perhaps a tier system or different categories of sustaining members could be developed which would increase both membership and revenues significantly while not causing losses among those organizations or members already enrolled.

The Committee feels that a lack of communication exists between the Board of Directors, Long Range Planning Committee, and/or other committees whose functions relate to the charge of the Ways and Means Committee. This inevitably hampers the deliberations of the Committee. The Committee recommends that the Executive Director's office act as a clearing house of pertinent information.

Report of the Committee on State and Provincial Participation

HERSHEL F. MORRIS, JR, *Chairman*
Louisiana Department of Agriculture, University Station, Box 16390-A, Baton Rouge, LA 70893

Other Members: H. B. Bradford; P. C. Brignac; P. Caudill; W. Cobb; M. Foster; R. Frank; A. Gardner; E. Hargesheimer; J. Hebert; T. L. Jensen; W. V. Kadis; J. Kapish; S. E. Katz; D. McDaniel; J. Padmore; P. R. Rexroad; M. Rhodes; R. Speth; G. Tichelaar; L. Torma

The Committee on State and Provincial Participation met on October 29, 1985. The main topic of discussion was the Terms of Reference for the Committee. Many of the charges under the current Terms of Reference have been met by the Committee or assumed by other AOAC committees.

In light of this fact, the Committee recommends that the directives for this committee, and all committees of AOAC, come from the Board of Directors or its representative, such as a committee on committees. The Board or committee on committees should charge and coordinate the actions of all AOAC committees and review their terms of reference to reduce overlapping of goals and objectives.

The Committee on State and Provincial Participation will continue to act as liaison between the AOAC office and state, provincial, and industrial laboratories. The Committee awaits specific direction from the AOAC Board of Directors on its future Terms of Reference.

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AOAC Meetings

June 16-18, 1986
Midwest Regional Section Meeting

Lincoln, Nebraska
Contact: Thomas Jensen
Nebraska Department of Agriculture
3703 South 14th Street
Lincoln, NE 68502
(402) 471-2176

June 24-25, 1986
Northeast Regional Section Meeting

Canisius College, Buffalo, New York
Contact: Gerald L. Roach
Food and Drug Administration
599 Delaware Avenue
Buffalo, NY 14202
(716) 846-4494

September 15-18, 1986
100th Annual International
Meeting & Exposition

The Registry, Scottsdale, Arizona
Contact: Margaret Ridgell
AOAC
1111 North 19th Street, Suite 210
Arlington, VA 22209
(703) 522-3032

April 27-30, 1987
12th Annual
Spring Training Workshop

The Skyline Ottawa Hotel
Ottawa, Ontario, Canada
Contact: Graham MacEachern
Agriculture Canada, Plant Products Bldg. #22
Ottawa, Ontario, Canada K1A 0C5
(613) 994-1991
Contact: James Lawrence
Health and Welfare Canada
Health Protection Branch, Tunney's Pasture
Ottawa, Ontario, Canada K1A 0L2
(613) 990-8459

September 14-17, 1987
101st Annual International
Meeting & Exposition

The Cathedral Hill Hotel
San Francisco, California
Contact: Margaret Ridgell, AOAC

August 29-September 1, 1988
102nd Annual International
Meeting & Exposition

The Breakers
Palm Beach, Florida
Contact: Margaret Ridgell, AOAC

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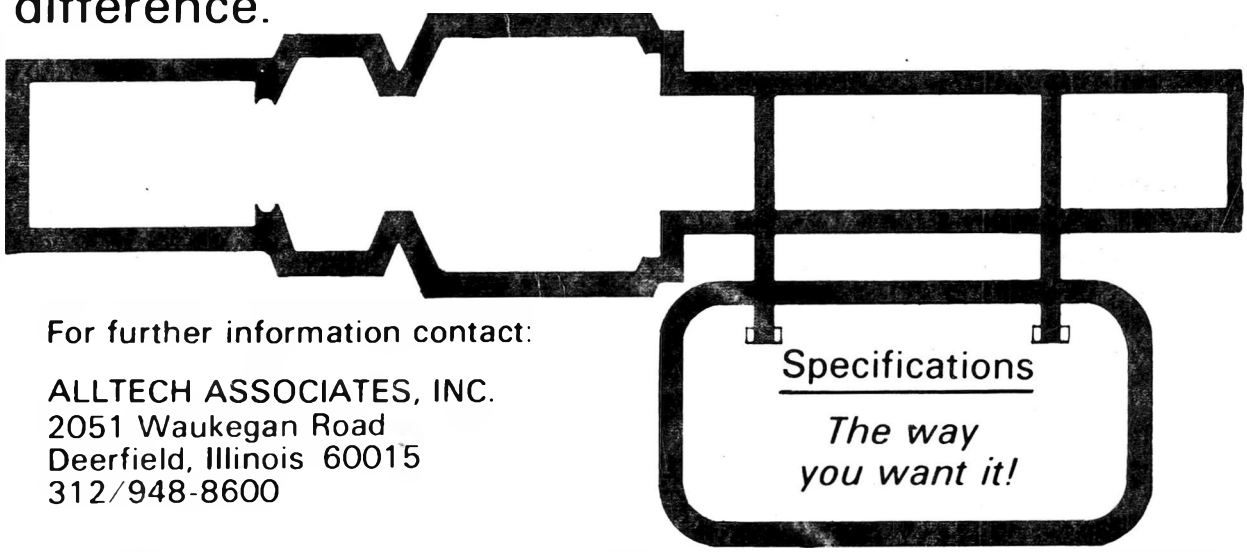

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