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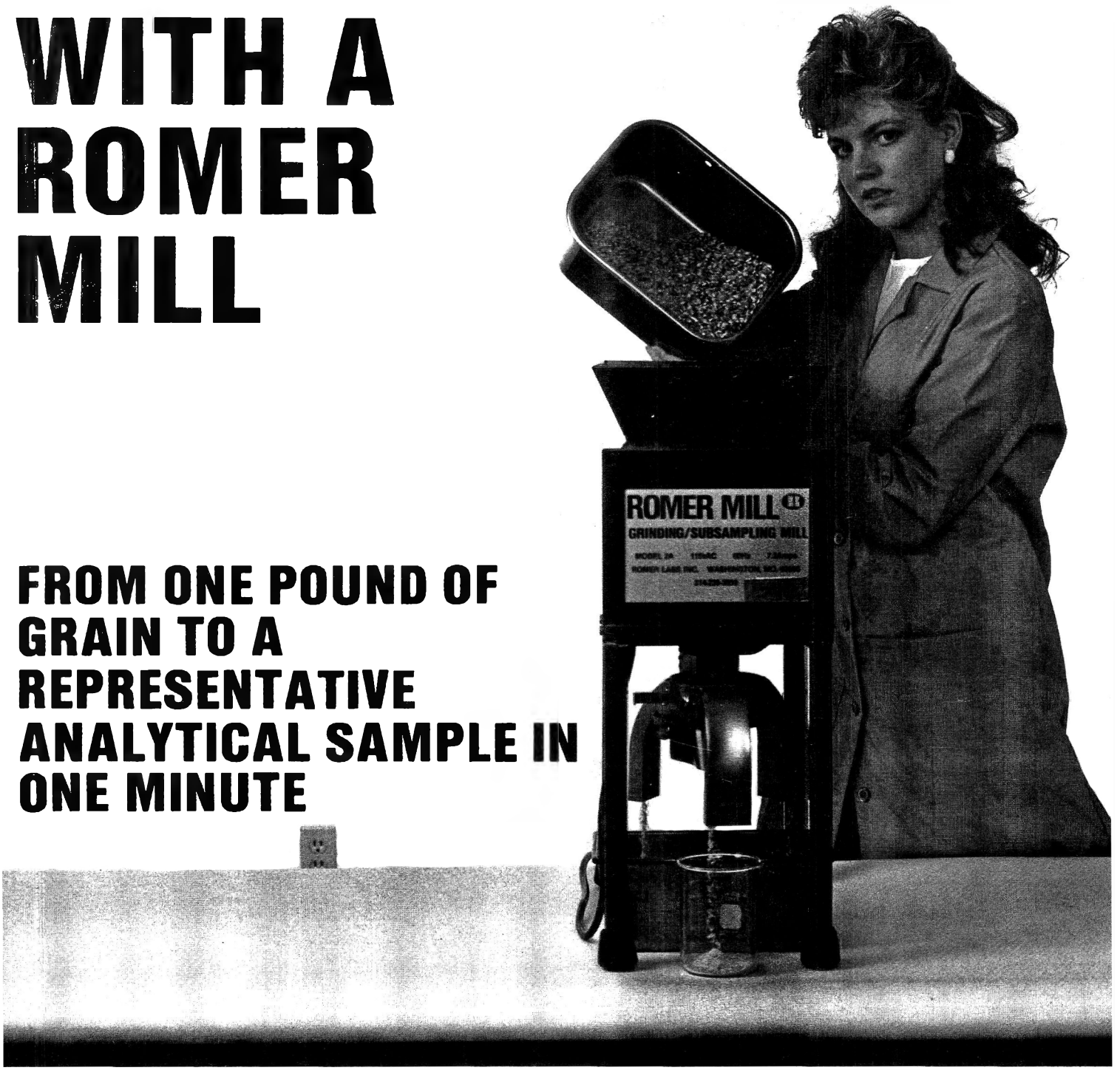

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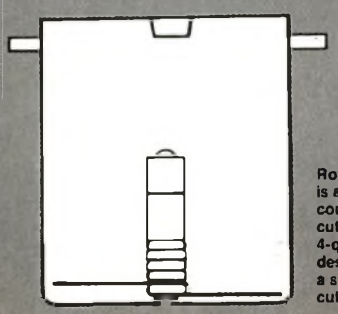

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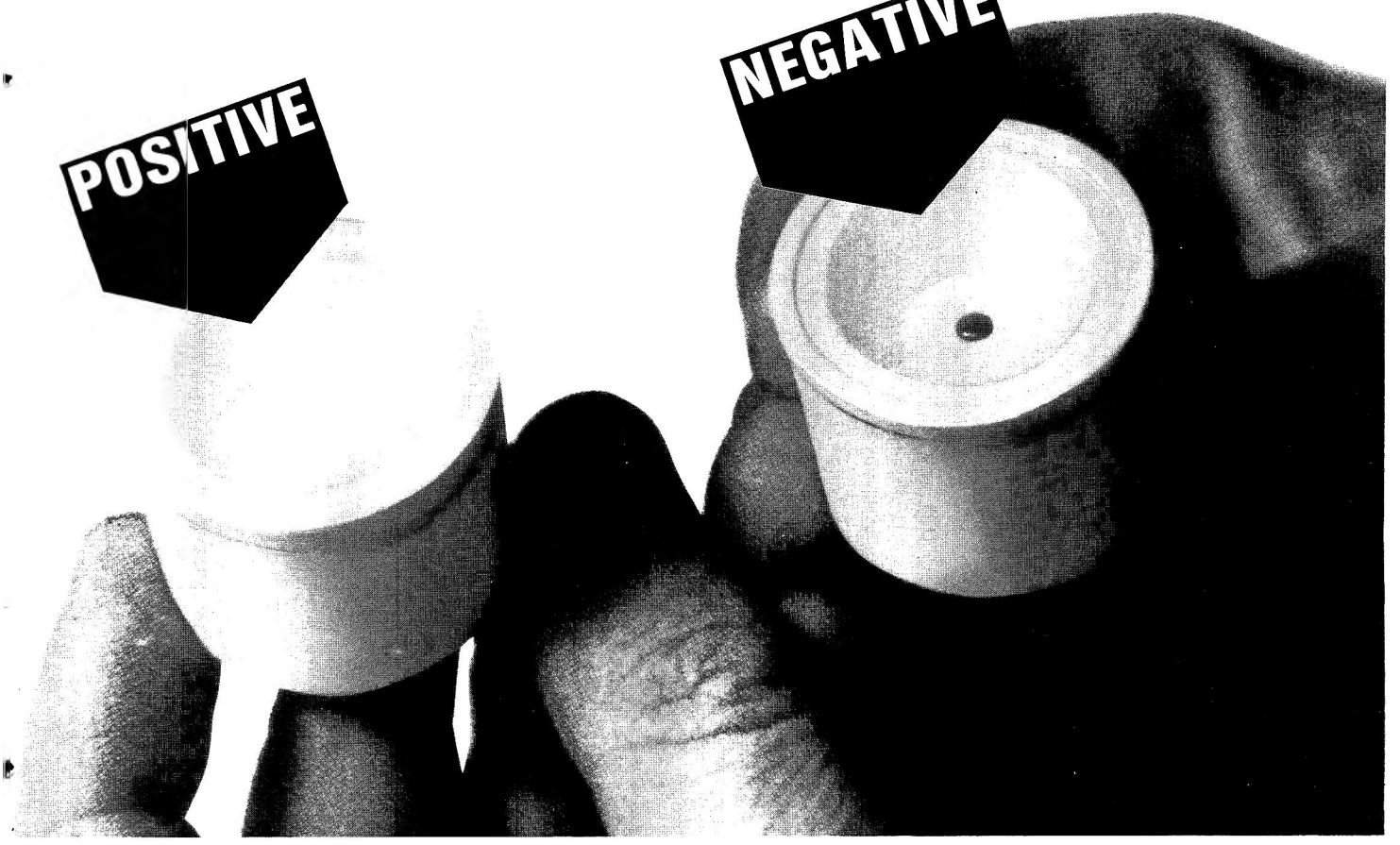

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●◆■ Penicillin N
●◆■ Methicillin
●◆■ Nafcillin
●◆■ Ticarcillin
●◆■ Penicillin V.
(benzathine)
(hydrabamine)
(potassium)
●◆■ Oxacillin
●◆■ Cloxacillin
(benzathine)
●◆■ Dicloxacillin
●◆■ Flucloxacillin
●◆■ Ampicillin
(trihydrate)
●◆■ Amoxicillin
(trihydrate)
●◆■ Piperacillin
●◆■ Hetacillin
●◆■ Carbenicillin
●◆■ Cephalothin
(Cephaloglycin)
●◆■ Cephapirin
●◆■ Cephapirin Benzathine
●◆■ Cephradine
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●◆■ Cephalexin
●◆■ Cephaloridine
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●◆■ Cefmenoxime
●◆■ Cefmetazole
●◆■ Cefonicid
●◆■ Cefoperazone
●◆■ Ceforanide
●◆■ Cefotaxime
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●◆■ Ceftezole
●◆■ Ceftizoxime
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●◆■ Cephamycin B
●◆■ Cephamycin C
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■ Oxytetracycline
■ Demeclocycline
■ Methacycline
■ Doxycycline
■ Minocycline

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■ Amikacin
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■ Tobramycin

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Erythromycin Gluceptate
Erythromycin Lactobionate
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Erythromycin Thiocynate
●◆■ Oleandomycin
●◆■ Tylosin
●◆■ Lincomycin
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Aminex HPLC columns are extremely useful for such biotechnology applications as monitoring cell metabolites, analyzing for specific biochemical compounds, and analyzing for radiolabeled compounds. Aminex carbohydrate analysis columns resolve simple and complex carbohydrates associated with glycoproteins, cell culture media, microbial metabolism, urine, and pharmaceutical (per USP procedures) matrixes. Compounds such as neutral sugars, *N*-acetylated sugars, radiolabeled sugars, alditols, and sugar alcohols are easily resolved and quantitated with the carbohydrate analysis columns. Bio-Rad Laboratories.
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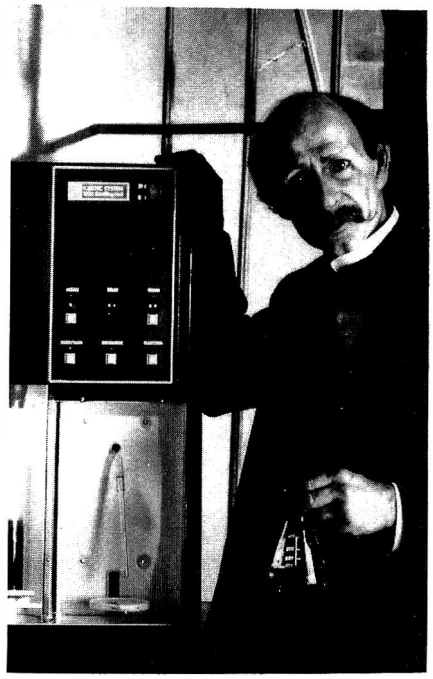
Nitrogen in Wastewater.



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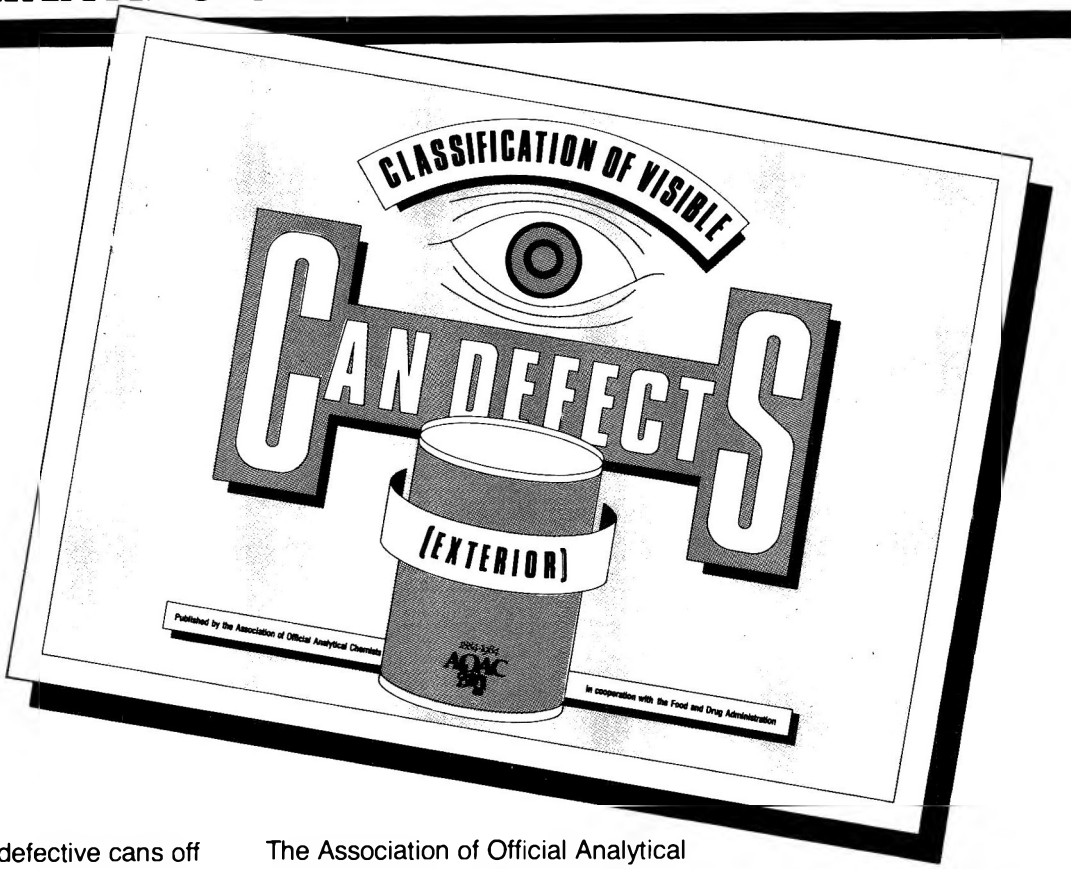
ELIMINATING CAN DEFECTS—THE FIRST STEP



How can we keep defective cans off the market?

Botulism and other forms of food poisoning can sometimes be traced to defective cans that have leaked and thereby allowed micro-organisms to enter food. Recognizing a can defect, doing something to correct the cause, and removing the defective cans from commerce will help prevent food poisoning outbreaks. The first step is to ensure that responsible personnel know how to identify defective cans.

The Association of Official Analytical Chemists, in cooperation with the Food and Drug Administration, has published a pamphlet that unfolds to a 24″ × 36″ chart, suitable for wall display, to help food industry personnel learn to identify can defects quickly. The chart uses a combination of photographs, easy-to-follow explanations, and color coding to illustrate can defects, classify them according to their degree of potentia hazard, and show what to look for in routine inspection of the finished product.

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

For copies, send order form and check to:

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BOOKS IN BRIEF

Evaluation of Programmes to Ensure Food Safety. Published by the World Health Organization, Distribution and Sales, 1211 Geneva 27, Switzerland, 1989. 47 pp. Price: Sw.fr. 9.-U.S. $7.20. ISBN 92-4-154247-0.

This book presents concepts, arguments, and suggested lines of action that respond to the need for better programs to ensure food safety. Addressed to national authorities, the book emphasizes the importance of evaluation as a managerial tool for identifying food safety problems and designing appropriate corrective programs. Throughout, an effort is made to help readers understand both the difficulty of implementing an effective food safety program and the advantages that a sound program can bring in terms of reduced economic losses and improved public health.

Guidelines for Predicting Dietary Intake of Pesticide Residues. Published by the World Health Organization, Distribution and Sales, 1211 Geneva 27, Switzerland, 1989. 24 pp. Price: Sw.fr. 8.-/U.S. $6.40. ISBN 92-4-154250-0.

Procedures are described that can be used by national authorities to predict the dietary intake of pesticide residues and thus reach conclusions concerning the acceptability of Codex Maximum Residue Limits from a public health point of view. Prepared in collaboration with the Codex Committee on Pesticide Residues, the guidelines respond to the need for a method of reaching reasonable assurance that the intakes of pesticide residues for different populations do not exceed safety limits.

Principles of Food Sanitation, Second Edition. By Normal G. Marriott. Published by VNR, 7625 Empire Dr, Florence, KY 41042-2978, 1989. 384 pp. Price: $49.95. ISBN 0-442-31807-3.

This second edition updates and expands discussion of food sanitation principles for dairy, meat, seafood, fruit, vegetable and beverage plants, and food service operations. The author has expanded the discussion of food regulations, pest control, sanitizers, and environmental pathogens. All chapters have been revised and a new chapter on beverage plant sanitation has been added. Includes a featured discussion of the hazard analysis critical control point (HACCP) concept and the development of a practical quality assurance and sanitation program.

Sonochemistry: Theory applications and Uses of Ultrasound in Chemistry. By Timothy J. Mason and J. Phillip Lorimer. Published by John Wiley & Sons, Inc., 1 Wiley Dr, Somerset, NJ 08875-1272, 1989. 252 pp. Price: $87.95. ISBN 0-470-21373-6.

The use of ultrasound in chemistry is an advancing field of study which could become as important as photochemistry, thermochemistry, or high pressure chemistry. It can be applied across the entire spectrum of chemical topics, from polymer science, through analytical and synthetic chemistry, to chemical physics, with the distinct advantage that its methodology is straightforward and the apparatus requirements are simple. This unified account covers the various facets of the topic, linking them to form a balanced approach for all chemists interested in starting, or continuing to study, the uses of ultrasound in chemistry. Written by two well-known sonochemists, for chemists, it brings together the theory and practice of sonochemistry in easily readable and assimilable form.

Chemiluminescence: Principles and Applications in Biology and Medicine. By A. K. Campbell. Published by VCH Publishers, Inc., Suite 909, 220 East 23rd St, New York, NY 10010-4606, 1988. 250 pp. Price: $196.00. ISBN 0-895-73501-6.

The text describes the occurrence, chemistry, and measurement of chemiluminescence, a phenomenon that together with the use of very sensitive light detectors has led to a number of biomedical applications and new concepts about disease processes. The author addresses the principles of using the reactions from fireflies and luminous bacteria to measure enzymes and metabolites, using calcium-sensitive proteins from luminous jellyfish to measure changes in calcium inside living cells, and measuring oxygen radicals and replacing radioactive labels in immunoassay by chemiluminescent compounds. The author also discusses the future applications in research and clinical laboratories.

Quadrupole Storage Mass Spectrometry. By Raymond E. March and Richard J. Hughes. Published by John Wiley & Sons, Inc., 1 Wiley Dr, Somerset, NJ 08875-1272, 1989. Price: $69.95. ISBN 0-471-85794-7.

The versatility, speed, and accuracy of the quadrupole ion trap represents a new era in the field of mass spectrometry. This volume brings together virtually all the pertinent information on the quadrupole ion trap, together with a detailed account of the underlying theory of its operation. The historical review outlines the development and characterization of the quadrupole ion trap. Quadrupole theory is presented with a clear, step-by-step mathematical treatment. Succeeding chapters present detailed treatments of the physics and chemistry of the ion trap, discuss infrared multiphoton dissociation of gaseous ions, review the theory and utilization of cylindrical ion traps, and detail the Finnigan MAT ion trap detector and the ion trap mass spectrometer.

CRC Handbook of Laboratory Safety, Third Edition. Edited by A. Keith Furr. Published by CRC Press, Inc., 200 Corporate Blvd, NW, Boca Raton, FL 33431, 1989. 464 pp. Price: $95.00 (U.S.); $112.00 (export). ISBN 0-8493-0353-2.

The third edition builds on the strength of earlier editions, but takes some new directions which make it an especially practical and useful reference tool. The book brings managers and laboratory personnel up-to-date on effective programs designed to meet the needs of new regulatory standards, including regulations in the areas outside the immediate operations of the laboratory. For example, laboratory operations must be performed in code complying facilities; hazardous waste disposal is now highly regulated; and right-to-know legislation covers laboratory personnel, and also the nearby community. Substantial new information on safe operations is also consid-

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ered. Health aspects of health and safety programs include AIDS, control of laboratory infections, recombinant DNA, carcinogenic effects, teratogenic effects, and medical surveillance programs.

Natural Products Isolation: Separation Methods for Antimicrobials, Antivirals, and Enzyme Inhibitors. By G. H. Wagman and R. Cooper. Published by Elsevier Science Publishers, PO Box 211, 1000 AE Amsterdam, The Netherlands, 1989. 618 pp. Price: $139.00/Dfl. 285.00. ISBN 0-444-87147-0.

The book encompasses the most recent progress made in the isolation and separation of natural products, including antibiotics, marine- and plant-derived substances, enzyme inhibitors, and interferons. The most recent separation methodology is described. Although there is a bias toward antibiotics, it was done because this is still the largest natural products area of research. The 14 chapters are all written by experts in their respective fields. The book has extensive isolation schemes and techniques, tables, figures, and chemical structures. In many instances, a short summary of the producing organism, brief chemical description and structure, and biological activity of the compounds is presented. Each chapter has an extensive bibliography and, where applicable, an appendix showing sources of materials and equipment. A detailed index is included.

Stereoselectivity of Pesticides: Biological and Chemical Problems. Edited by E. J. Ariëns, J. J. S. van Rensen, and W. Welling. Published by Elsevier Science Publishers, PO Box 211, 1000 AE Amsterdam, The Netherlands, 1988. 544 pp. Price: $171.00/Dfl. 325.00. ISBN 0-444-42853-4.

The growing concern about the hazards for humans and their environment due to exposure to chemicals includes attention to stereoselectivity in bioactive agents such as pesticides, drugs, etc. Mixtures of stereoisomers often contain 50% inactive material. Written for workers in the field of pesticides, the book contains concise and basic information on stereoselectivity as a notable source of problems. Attention is paid to the involvement of stereoselectivity in important classes of pesticides as well as to pheromones and compounds derived from insect hormones with potential use in crop protection. Fundamentals of stereochemistry, stereoselective analytical methods, stereoselective synthesis, and stereochemical aspects of the design of new compounds are also discussed.

Basic Food Microbiology, Second Edition. By George J. Banwart. Published by VNR, 7625 Empire Dr, Florence, KY 41042-2978, 1989. 773 pp. Price: $54.95. ISBN 0-442-22120-7.

The completely revised and unabridged second edition features new information on biotechnologies and foodborne illnesses, such as listeriosis and *Campylobacter jejuni*. Also new are flowsheets for food processes, and flow diagrams that depict the production of dairy products and beer.

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AOAC

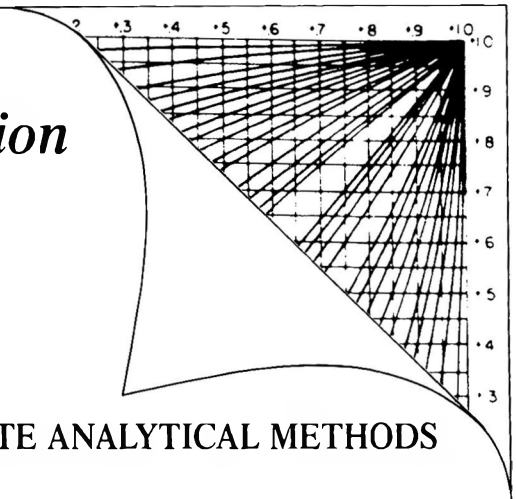
AWARDS NOMINATION DEADLINES

Letters in Support of AOAC Fellows Awards — The deadline is **February 15** of each year.

Harvey W. Wiley Scholarship Nominations —
A junior and senior year scholarship of $500 per year awarded annually to sophomores majoring in scientific areas of interest to AOAC. Each year, **May 1** is the nomination deadline. The award winner is announced about six weeks later.

Harvey W. Wiley Award for the Development of Analytical Methods —
A $2,500 annual award to an outstanding scientist or scientific team for analytical contributions in an area of interest to AOAC. Nominations will be accepted year-round. Those received before **December 1** of any year will be eligible for the following four years' awards. Eligibility may be extended an additional four years by written request of the nominator.

For more information contact: Administrative Manager, AOAC, Suite 400-J, 2200 Wilson Blvd., Arlington, Virginia 22201-3301 USA or phone (703) 522-3032.



AOAC Announces the Publication of an Indispensable Statistical Reference Book

USE OF STATISTICS TO DEVELOP AND EVALUATE ANALYTICAL METHODS

Grant T. Wernimont, Author
William Spendley, Editor

With the aid of this manual, the analytical investigator can, with a knowledge of only simple statistical procedures, use the experimental designs and techniques described to determine and evaluate assignable causes of variability.

The book reviews the basic operations in the process of making measurements, offers suggestions for planning experimental work so that appropriate statistical methodologies can be used to interpret the results, includes a number of experimental plans for developing and modifying analytical procedures, and discusses evaluation of the data.

Other features include scores of specific statistical analyses of real-life data, many useful statistical tables, and very complete references.

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

Contents

1. Introduction
   - AOAC and Collaborative Studies
   - Organization and Procedures for Collaborative Studies
   - Selection of Methods of Study
   - Types of Interlaboratory Study
   - Need for This Manual
2. The Measurement Process
   - What is Measurement?
   - Measurement as a Relationship Between Properties
   - 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 an Analytical Process
   - Interlaboratory Experiments
   - Objectives for Interlaboratory Study
   - 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

Appendixes: Tables, Statistical Computations, Glossary. Index.

1985. xvi + 183 pages. Softbound. ISBN 0-935584-31-5.

Price—Members: $49.55 in U.S., $54.55 outside U.S. Nonmembers: $54.50 in U.S., $59.50 outside U.S. Prices include handling and shipping.

To obtain this book, send order and check or credit card information (VISA or MasterCard number, expiration date, signature, date) with your name and address to:

AOAC, 2200 Wilson Blvd., Ste. 400-J, Arlington, VA 22201-3301 USA (703) 522-3032, FAX (703) 522-5468

(*U.S. funds on U.S. banks only*)

FOR YOUR INFORMATION

Meetings

November 1, 1989: Eastern Ontario-Quebec AOAC Regional Section Meeting. Contact: Gilles Paillard, Quebec Dept of Agriculture, 2700 Rue Einstein C2 72, Sainte Foy, PQ G1P 3W8, Canada, telephone 418/642-2561.

November 1–2, 1989: Central AOAC Regional Section Meeting. Contact: Georgia Markakis, Michigan Dept of Agriculture, 1615 South Harrison Rd, East Lansing, MI 48823, telephone 517/337-5040.

November 7, 1989: Mid-Canada AOAC Regional Section Meeting. ABI Biotechnology Institute, Winnipeg, Manitoba, Canada. Contact: Marc Duguay, Manitoba Research Council, 810 Phillips St, Box 1240, Portage la Prairie, MB R1N 2J9, Canada, telephone 204/857-7861.

November 15, 1989: New York-New Jersey AOAC Regional Section Meeting, Busch Student Center, Rutgers University, New Brunswick, NJ. Contact: Jeff Singer, Pall Corp., 30 Seacliff Ave, Glencove, NY 11542, telephone 516/671-4000.

November 13–14, 1989: AOAC Short Course—Quality Assurance for Analytical Laboratories, Arlington, VA. Contact: AOAC Short Courses, Suite 400, 2200 Wilson Blvd, Arlington, VA 22201-3301, telephone 703/522-3032.

February 5–8, 1990: Fourth International Symposium on Biological and Environmental Reference Materials, Sonesta Village Hotel, Orlando, FL. Contact: Wayne R. Wolf, B311 Chemistry Building, NIST, Gaithersburg, MD 20899, telephone 301/975-2030.

March 5–9, 1990: 41st Pittsburgh Conference & Exposition on Analytical Chemistry and Applied Spectroscopy, Jacob K. Javits Convention Center, New York, NY. Contact: Pittsburgh Conference, Dept CFP, 300 Penn Center Blvd, Suite 332, Pittsburgh, PA 15235.

April 3–5, 1990: Anatech '90—Second International Symposium on Applications of Analytical Techniques to Industrial Process Control, Leeuwenhorst Congress Centre, Noordwijkerhout, The Netherlands. Contact: Willem E. van der Linden, Laboratory for Chemical Analysis-CT, University of Twente, PO Box 217, NL-7500 AE Enschede, The Netherlands, telephone (53) 892629.

May 1990: Northeast AOAC Regional Section Meeting. Contact: Jon Schermerhorn, New York State Dept of Agriculture and Markets, 1220 Washington, Albany, NY 12235, telephone 518/485-8098.

May 15–18, 1990: Second International Symposium on the Analysis of Anabolizing and Doping Agents in Biosamples, State University of Ghent, Belgium. Contact: C. Van Peteghem, Pharmaceutical Institute, State University of Ghent, Harelbekestraat 72, B-9000 Ghent, Belgium, telephone 3291 21.89.51 ext. 235.

June 7–9, 1990: International Conference on Chirality, Sheraton Resort, Cancun, Mexico. Contact: Mary Jo Richards, Dept of Chemistry, University of Missouri-Rolla, Rolla, MO 65401, telephone 314/341-4429.

September 10–13, 1990: 104th AOAC Annual International Meeting and Exposition. The Clarion Hotel, New Orleans, LA. Contact: AOAC Meetings Department, Suite 400, 2200 Wilson Blvd, Arlington, VA 22201-3301, telephone 703/522-3032.

New Sustaining Members

AOAC welcomes the following new private sustaining members: Federation of Migros Cooperation, Zurich, Switzerland; Unilever Research U.S., Inc., Edgewater, NJ.

Permanent Numbers Assigned to Methods for 15th Edition

Methods in the 15th edition of *Official Methods of Analysis* will have permanent numbers that will continue as long as the method retains official status.

Publication of the 15th edition in early 1990 will institute this first major change in method numbering since the decimal system was introduced in 1945.

Until now, the number designations of methods could change with each edition as new methods and chapters were added and previous methods were revised or replaced.

The development of a permanent numbering scheme for official methods has been in the works since October 1976. The Publications Planning Committee of the Editorial Board was asked to devise such a system, and put forth several suggestions. But by the time the 13th edition was published in 1980, the Committee had not agreed on an approach.

In the 1980 preface, Editor William Horwitz stated, "Users expressed a desire for a system that will keep the same reference number for a given method from edition to edition. A practical system that will maintain the continuity of the numbering system and the organizational structure of the methods within a chapter has not yet been devised."

The Publications Planning Committee was subsequently disbanded, but a subgroup of the Editorial Board continued working. Throughout the deliberations, the group addressed these issues: adaptability of the proposed scheme to incorporate new methods, combinations of methods, and changes in existing methods; form of the number; procedure for renumbering existing methods, figures, and tables; procedure for handling cross references; and effect of a new system on the cost of production.

Although no system was completely satisfactory, several elements from diverse schemes were eventually selected: only one number would be assigned to a given method, serving only as a citation number; new methods would be assigned the next consecutive number at the time of adoption; chapters could be organized in any appropriate manner without regard for number sequence; and methods would be located by reference to number or subject indexes.

The basis of the new, permanent number: the year of adoption.

For example, 989 is assigned to a method adopted at the 1988 annual meeting and appearing for the first time in "Changes in Official Methods of Analysis" in 1989. The next digits are simply sequential for the methods adopted that year.

The newly adopted liquid chromatographic method for azinphos-methyl in pesticide formulations has the number **989.01.** The immunodiffusion screening method for motile *Salmonella* has the number **989.13.**

Permanent numbers in *Official Methods of Analysis* are important and desirable for several reasons.

Convenience. Users don't have to keep track of changing numbers for unchanging methods, and numbers don't have to be changed when chapters or volume(s) are reformatted in more usable arrangements.

Consistency for regulatory, legal, and reference citations. AOAC methods are cited worldwide in thousands of documents—state, provincial, and federal laws, definitions and standards of identity, public and private specifications and contracts.

Consistency for electronic databases.

Reduced error of citation. As different source documents are used in citing AOAC methods, correct citation will be assured.

The one drawback to the new scheme is the main reason why no system was completely satisfactory. Analysts most familiar with the Book of Methods know the chapter sequence and the arrangement of methods within a chapter, and know that the present number, e.g., **25.034,** identifies the chapter and location of the method.

The new permanent number will give no clue to the location of a method in the book. Researchers will need to consult a number index to locate the correct page for the method.

As an additional aid to the researcher, many of the cross references to apparatus and reagents described in methods in other chapters have been eliminated. This information has been brought back into each method description to allow more methods to "stand alone."

AOAC/Europe Regional Section Formed

AOAC's first Regional Section outside North America is being organized. Over 70 persons from 17 countries, representing four international organizations, recently met in Almere, The Netherlands, to establish the AOAC/Europe Regional Section.

The section will cover all of Europe and the Mediterranean countries.

The creation of the section is the culmination of many years of work by Margreet Lauwaars, AOAC's European Representative, and Ben Borsje of Amsterdam, The Netherlands, AOAC's first Associate Referee outside North America and the immediate past Chairman of AOAC's International Coordination Committee.

Paul Beljaars, a chemist with the Food Inspection Service, Maastricht, The Netherlands, was elected Regional Section president. "One of the main things we want to do is have contact with people in food chemistry and food microbiology," he said, "and to exchange knowledge in general about science and business."

"We want to give support to their work," said Beljaars, "and the way AOAC operates is very effective."

The AOAC/Europe meeting was held at the offices of Boehringer Mannheim, and attendees were welcomed there by J. P. Lorenz.

After presentations by Thomas Layloff, AOAC President-elect, on AOAC, and by Borsje on the history of Europeans' interest in AOAC activities, bylaws were approved and nine executive committee members were elected.

The meeting was followed by a technical seminar on immunochemistry with presentations by G. Wolters, Organon Diagnostics (NL); C. (Kit) Hitchcock, University of Surrey (UK) (recently retired from Unilever); and E. P. Martlbauer, University of Munich (FRG).

The following day, at a meeting hosted by the Department of Health, Welfare and Culture in Rijswijk, The Netherlands, the executive committee elected the following officers:

Paul Beljaars, President;

Ellen deVries, Duphar BV, Weesp, The Netherlands, Secretary-Treasurer;

Juan Sabater, Barcelona, Spain, Vice President.

Other members of the executive committee include: Anne-Lise Malsker, Danochemo A/S, Ballerup, Denmark; Roger Wood, Ministry of Agriculture, Fisheries and Food, London, U.K.; Claude Bourgeois, Societe Produits Roche, Fontenary Sous Bois, France; Toni Rihs, Swiss Federal Research Station for Animal Production, Posieux, Switzerland; B. King, Laboratory of the Government Chemist, Teddington, Middlesex, U.K.; and Maire C. Walsh, State Laboratory, Dublin, Ireland.

"Every country in Europe has its own chemical organization, with not much contact among them," said Beljaars. "AOAC can be the glue for food analysis and for microbiological analysis throughout Europe, especially with the formation of the European Community in 1992."

The Section got off to a very successful start. The 1990 meeting of AOAC/Europe will be held in Brussels.

New Poster on Flexible Packages to Help Spot Defects

AOAC has published a pictorial poster on flexible packages.

The 24″ × 36″ full-color poster, named "Classification of Visible Flexible Package Defects (Exterior)," has been published by AOAC in cooperation with the National Food Processors Association (NFPA) and the Food and Drug Administration. It is the result of a 4-year study by NFPA and follows the success of the poster on classification of visible defects in cans, first published by AOAC in 1984.

The cans poster sold by the thousands to fast food chains, grocery stores, warehouses, food banks, military institutions, state and federal food inspectors, and hundreds of others involved in food handling.

According to Cleve Denny, an NFPA microbiologist in Washington, DC, the poster features the four types of packages: paperboard packages and flexible pouch packages on side one, plastic packages with heat-sealed lids, and plastic cans with double-seamed metal ends on side two.

Denny said the poster has been published by AOAC simultaneously with NFPA's publication of a "Flexible Package Integrity (FPI) Bulletin."

The NFPA project on cans started in 1980 following an outbreak of lethal food poisoning in Britain that was tied to leakage problems with canned foods. At that time, NFPA established the Container Integrity Task Force, a 90-person team chaired by the head of American National Can Company, Arnold Kopetz.

"The Task Force worked for 4 years, when they produced the cans poster, among other products," said Denny, who served as Secretary. "At the end of the project, we knew a lot about cans, but we realized we didn't know a lot about flexible packages."

So in 1984, NFPA formed the Flexible Package Integrity Committee, an 80-person team co-chaired by Kopetz and Charles Sizer of Tetra Pak Inc.

That Committee studied three areas: defect definitions and classifications, good manufacturing practices, and test methods.

The poster on flexible packages defines and classifies defects. Cost of "Classification of Visible Flexible Package Defects (Exterior)" is $72.00 per package of 10 posters, $20.00 for a simple poster, plus shipping and handling costs: $4.00 in U.S., $8.00 outside U.S. per poster or package of posters.

AOAC Books in International Exhibits

AOAC's books and *Journal* are being exhibited at overseas posts of the U.S. Information Agency (USIA), selected International Book Fairs, and other traveling international exhibits.

The series of exhibits are a cooperative effort between USIA and the

Council of Engineering and Scientific Society Executives, of which AOAC is a member.

The exhibit will be split into two sections: Twenty-six exhibits will be rotated among USIA posts; permanent journal exhibits will be set up at about 70 USIA libraries and cultural centers in major cities worldwide.

AOAC will exhibit its *Journal* and the following books: *Use of Statistics to Develop and Evaluate Analytical Methods; Bacteriological Analytical Manual; Quality Assurance Principles for Analytical Laboratories; Statistical Manual of the AOAC;* and *Official Methods of Analysis, 14th edition.*

The exhibit schedule includes:

Posts	Start Dates
Hong Kong	July 1, 1989
Taipei, Taiwan	Sep. 1, 1989
Rangoon, Burma	Dec. 1, 1989
Bangkok, Thialand	July 1, 1989
Jakarta, Indonesia	Oct. 1, 1989
Kuala Lumpur, Malaysia	Jan. 1. 1990
(Feb.-July 1990 displays at leading bookstores, libraries, and universities. *Malaysia Book Fair* Aug. 1990)	
Manila, Philippines	Sep. 1, 1989
Beijing, China	Jan. 1, 1990
Montevideo, Uruguay	July 1, 1989
Port of Spain, Trinidad	Jan. 1, 1990
Santiago, Chile	Apr. 1, 1990
Rio de Janeiro, Brazil	July 1, 1989
(six months showing at leading bookstores, academic institutions, and national libraries)	
Caracas, Venezuela	July 1, 1989
Tegucigalpa, Honduras	Sep. 1, 1989
(exhibit at major science seminars to be held late Sep. and Oct. in Tegucigalpa and San Pedro Sula with attendance by scholars from Honduras, Central America, the United States, and Europe. Worldnet inter-actives planned for event in Tegucigalpa)	
Georgetown, Guyana	Jan. 1, 1990
Guatemala City, Guatemala	July 1, 1989
Lima, Peru	Oct. 1, 1989
Kingston, Jamaica	Jan. 1, 1990
Oslo, Norway	June 15, 1989
(exhibit at major *North Norwegian Trade Fair* June 1989)	
Stockholm, Sweden	Sep. 1, 1989
Rome, Italy	Nov. 15, 1989
(displays at Rome and Bologna universities, Rome American Studies Center, and Sassari University on the occasion of the biennial meeting of the Italian Assn of North American Studies Nov. 1989)	
Belgrade, Yugoslavia	Sept. 15, 1989
(centerpiece of the American stand at the *Belgrade International Book Fair* Oct. 1989)	
Bucharest, Romania	Dec. 1, 1989
Valletta, Malta	Sep. 15, 1989
(exhibition at 1989 *Malta International Book Fair* Oct. 23–29, 1989)	
Ankara, Turkey	Nov. 15, 1989
The Hague, The Netherlands	Jan. 15, 1990
Athens, Greece	Jan. 1, 1990
(three months showing at multiple scientific research institutions in Athens, Thessaloniki, Patras, and Iráklion, Crete)	
Helsinki, Finland	June 1, 1990
(exhibit at "America Days" in city of Lappeenrantha)	
Prague, Czechoslovakia	Aug. 15, 1990
Islamabad, Pakistan	June 15, 1989
(six month showing at major scientific research centers—Islamabad, Karachi, Lahore, and Peshawar)	
Cairo, Egypt	Jan. 1, 1990
(*Cairo International Book Fair*, Jan. 1990)	
New Delhi, India	July 1, 1989
(various book fairs and book exhibitions in Madras, Bombay, Calcutta, and the *Delhi World Book Fair* Feb. 1990)	
Khartoum, Sudan	Sep. 15, 1989
Riyadh, Saudi Arabia	Dec. 15, 1989
(in-country tours)	
Safat, Kuwait	Sep. 1, 1989
Dhaka, Bangladesh	Nov. 1, 1989
Amman, Jordan	Sep. 1, 1989
San'a, Yemen	Nov. 15, 1989
Colombo, Sri Lanka	Nov. 1, 1989
(exhibit at the 45th meeting of the Sri Lanka Assn for the Advancement of Science, Dec. 1–31, 1989)	
Abu Dhabi, UAE	Feb. 1, 1990
Kathmandu, Nepal	Aug. 1, 1989
Tunis, Tunisia	Nov. 1, 1989
Muscat, Oman	Nov. 1, 1989
Manama, Bahrain	Feb. 1, 1990
Damascus, Syria	July 1, 1989
(*Damascus Book Fair*	Sep. 1989)
Mogadishu, Somalia	Sep. 1, 1989
Harare, Zimbabwe	Feb. 1, 1990
Linlongwe, Malawi	Sep. 1, 1989
Accra, Ghana	Feb. 1, 1990
Abidjan, Ivory Coast	Aug. 1, 1989
Algiers, Algeria	Dec. 15, 1989
Douala, Cameroon	Aug. 1, 1989
Lusaka, Zambia	Dec. 1, 1989
Reserved for major Book Fairs, Spring 1990 (*Leipzig International Book Fair*, Berlin, E. Germany, March 1990)	
Reserved for special East European tour	

UPCOMING AOAC ANNUAL MEETING

104th AOAC Annual International Meeting in New Orleans, Louisiana September 10-13, 1990

INSTRUCTIONS TO AUTHORS

Scope of Articles and Review Process

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

General Information

Contributed manuscripts accepted for publication after peer review are subject to a charge of US$40 per printed page. Payment is not a condition of publication, however, and waivers are granted on receipt of a written request to the Managing Editor by an administrative officer of the author's institution.

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

1. Write in clear, grammatical English.
2. To Managing Editor, AOAC, Suite 400, 2200 Wilson Blvd, Arlington, VA 22201-3301 USA, submit typewritten original plus 3 photocopies (1 side only, white bond, 8½ × 11 in. [21½ × 28 cm]) of complete manuscript in order as follows—1. Title page; 2. Abstract; 3. Text (introduction, method or experimental, results and/or discussion, acknowledgments, references); 4. Figure captions; 5. Footnotes; 6. Tables with captions, one per page; 7. Figures.
3. Suggest in a covering letter the names of at least 4 qualified reviewers, i.e., individuals engaged in or versed in research of the type reported.
4. **DOUBLE SPACE all typed material.** Manuscripts not double spaced will be returned for retyping. Do not right justify or use proportional spacing; avoid hyphenation.
5. Use letter quality printer for word-processed manuscripts; manuscripts prepared on dot matrix printers of less than letter quality may be refused.

Format and Style

1. **Title page** (separate sheet, **double spaced**): Title of article, authors' names (full first, middle initial if any, full last), authors' addresses including mail codes.
2. **Abstract** (separate sheet, **double spaced**): ≤200 words. Provide specific information, not generalized statements.
3. **Text** (consecutive sheets, **double spaced**):

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

   Method or Experimental. Consult recent issue of *Journal* for proper format. Separate special reagents/apparatus from details of procedure and list in sections with appropriate headings; list in generic and performance terms, avoid use of brand names. (Common reagents/apparatus or those which require no special treatment need not be listed separately.) Place detailed operations in separate sections with appropriate headings (e.g., Preparation of Sample, Extraction and Cleanup). Include necessary calculations; number of significant figures must reflect accuracy of method. Use metric units for measurements of quantities wherever possible. Write *Method* (recommendation for use of specific method) in imperative voice ("Add 10 mL . . . Heat to boiling . . . Wash flasks"); write *Experimental* (description of laboratory experiment) in passive or active voice ("Ten mL was added . . . We heated to boiling . . . Flasks were washed"). Note hazardous and/or carcinogenic chemicals.

   Results/Discussion. Cite tables and figures consecutively in text with Arabic numerals. *Do not* intersperse tables and figures in text.

   Acknowledgments. Give brief thanks (no social or academic titles) or acknowledge financial aid in this section.

   References. Submitted papers or unpublished oral presentations may not be listed as references; cite them in text as unpublished data or personal communications. Cite all references to previously published papers or papers in press in numerical order in text with number in parentheses on line (*not* superscript). List references numerically in "References" in *exactly* (arrangement, punctuation, capitalization, use of ampersand, etc.) styles of examples shown below or see recent issue of *Journal* for less often used types of entries. Follow *Chemical Abstracts* for abbreviations of journal titles.

   JOURNAL ARTICLE REFERENCE

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

   BOOK CHAPTER REFERENCE

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

   BOOK REFERENCE

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

   OFFICIAL METHODS REFERENCE

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

4. **Figure captions** (separate sheet(s), **double spaced**): Designate all illustrations, including schemes, as figures and include caption for every one. Identify curves (See **Figures**) and include all supplementary information in caption rather than on face of figure. Spell out word Figure.
5. **Footnotes** (separate sheet, **double spaced**): Avoid use of footnotes to text. Include "Received . . . Accepted . . ." line; location/date of presentation, if appropriate; present address(es) of author(s); identification of corresponding author, if not senior author; proprietary disclaimers; institution journal series numbers.
6. **Tables** (one per page, **double spaced**): Refer to recent issue of *Journal* for proper layout and style, especially use of horizontal lines. Do not draw in vertical lines. Include descriptive title sufficient that table stands alone without reference to text. Provide heading for *every* vertical column. Abbreviate freely; if necessary, explain in footnotes. Indicate footnotes by lower case superscript letters in alphabetical order. Do not use one-column tables; rather, incorporate data in text.
7. **Figures:** The *Journal* does not publish straight line calibration curves; state such information in text. Do not duplicate data in tables and figures. Submit original drawings or black/white glossy photographs with original manuscript; photocopies are acceptable only for review. Prepare drawings with black India ink or with drafting tape on white tracing or graph paper printed with nonreproducible green ink. Use a Leroy lettering set, press-on lettering, or similar device; use type at least 2 mm high to allow reduction to page or column size. Identify ordinate and abscissa and give value in *Journal* style (e.g., "Wavelength, nm," "Time, min"). Label curves with letters or numbers; avoid all other lettering/numbering on face of figure (see **Figure captions**). Identify each figure on back with number and authors' names.

Miscellaneous

Abbreviation for liter is L; abbreviation for micron is μm. Do not italicize common Latin expressions such as et al. and in vitro; for nomenclature of spectrophotometry, gas chromatography, and liquid chromatography, follow practice of American Society for Testing and Materials (in particular, do not use "high performance," "high pressure," or the abbreviation "HP" with "liquid chromatography").

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MYCOTOXINS

- Comparison of Two ELISA Screening Tests with Liquid Chromatography for Determination of Aflatoxins in Raw Peanuts—by *Joe W. Dorner* and *Richard J. Cole*

FOOD AND DRUG ADMINISTRATION PESTICIDE PROGRAM

RESIDUES IN FOODS-1988

This is the second annual report in a series about the Food and Drug Administration's (FDA) pesticide residue monitoring program. The first report, which was published in the November/December 1988 issue of the **Journal of the Association of Official Analytical Chemists,** *described the FDA program and presented the results of monitoring for pesticide residues in the U.S. food supply for Fiscal Year (FY) 1987 (October 1, 1986, through September 30, 1987). The present paper explains the changes made in the FDA program since 1987, reports the results of FDA's pesticide monitoring for FY88 (October 1, 1987, through September 30, 1988), and compares some of the FY88 findings with those of FY87.*

The Environmental Protection Agency (EPA) registers or approves the use of pesticides and, if use of a pesticide may result in residues in foods, establishes tolerances. (A tolerance is the maximum amount of a residue expected in a food when a pesticide is used according to the label directions, provided that the level does not present an unacceptable health risk.) FDA is responsible for enforcing tolerances on food shipped in interstate commerce (except for meat and poultry, for which the U.S. Department of Agriculture is responsible), and, as part of its activities, determines the incidence and levels of various pesticides in domestically produced and imported foods. About 300 pesticide chemicals have food and/or feed tolerances in the United States.

FDA's monitoring of the food supply for over 25 years has shown that above-tolerance residues are rarely found. Violations more commonly involve commodities that contain small amounts of pesticides for which no tolerance has been set.

Residues of some pesticides that are no longer approved for agricultural use are unavoidably present at very low levels in both domestic and imported foods due to their persistence as environmental contaminants. In such situations, FDA establishes action levels, based on EPA recommendations, for enforcement purposes. These action levels specify the level below which FDA may exercise its discretion not to take enforcement action. FDA issued a notice in the February 19, 1988, *Federal Register* which stated that its action levels are not binding on the courts, the public (including food producers), or the agency (including individual FDA employees). Thus, if a food (or feed) contains a pesticide residue below the action level for that substance, FDA, through the Department of Justice, is not precluded from initiating seizure, injunction, or prosecution. At the same time, if a food contains a pesticide residue above the action level for that chemical, FDA is not necessarily required to take legal action. Whether legal action is taken in a given case will depend on several factors, such as the extent of the contamination, the strength of the evidence of contamination, and the risk to public health.

MONITORING PROGRAMS

Regulatory Monitoring

FDA examines raw produce as well as ready-to-eat foods.

To fulfill its mandated responsibilities, FDA collects samples from individual lots of domestically produced and imported foods and analyzes them for pesticide residues. The analytical methods used to measure the residues are generally capable of determining levels well below tolerance values. When violative residues are found in domestic samples, FDA can initiate various sanctions such as a seizure or injunction. For import samples, shipments may be detained at the port of entry when illegal residues are found.

Samples, both domestic and import, are classified as either "surveillance" or "compliance." Most samples collected by FDA are in the first category, that is, they are collected without suspicion that a particular shipment contains illegal residues. Compliance samples are collected and analyzed as follow-up to the finding of an illegal residue or when other evidence indicates that a residue problem may exist in that shipment.

When a shipment of an imported food from a particular grower or shipper is found to contain illegal pesticide residues, FDA may invoke automatic detention for future shipments of the food from that same source. While automatic detention is in effect, the importer is responsible for having each shipment of the commodity in question analyzed and certified by a private laboratory (most of them are in the United States) to be free of violative levels of the specific residue(s) in question.

Prior to 1988, the automatic detention of foods due to illegal pesticide residues was known as the certification requirement. In 1988, FDA revised the criteria for imposing automatic detention. Now, automatic detention may be invoked based on the finding of *one* violative sample if the product contains violative levels of a pesticide residue and there is reason to believe that the same situation will exist in future lots during the same shipping season for a specific shipper, grower, geographic area, or country.

For fresh produce, automatic detention remains in effect for all subsequent lots of the same produce for the rest of the growing season unless the grower/shipper demonstrates that the residue problem no longer exists or that the produce to be shipped originated in fields that were not treated with the pesticide in question.

FDA/State Cooperation.—Local or regional FDA personnel may interact with state departments of food and agriculture, health departments, plant boards, milk boards, or other similarly named divisions within a state's governmental organization. The types and amount of interaction between FDA and individual states vary, but nearly all states (except those few for whom agriculture is a minor concern and who therefore have a limited formal pesticide program) have some cooperative effort. A number of states meet regularly with FDA personnel to share information, discuss mutual problems, and, in some cases, to coordinate state/FDA sampling plans. About half of the states currently supply residue data to their FDA regional counterparts, and some exchange residue data with FDA at set intervals. Several states emphasize testing of milk/dairy products, grains, or seafoods if those commodities are of major economic interest, and this allows FDA to concentrate its efforts on other commodities. Instances of violative samples are immediately brought to the attention of the other party, and if FDA determines that a lot is violative, the state may assist by embargoing the violative shipment.

Six states (California, Florida, Massachusetts, New York, Virginia, and Wisconsin) presently contribute their monitoring data to "Foodcontam," a system supported by FDA via contract (with Mississippi State University) to compile state data on pesticide residues in foods. In 1988, data reflecting several thousand samples were received from these states. The overall findings are reviewed and considered by individual FDA Districts in the development of pesticide monitoring plans and to uncover potential problem areas.

This report, as in 1987, covers only FDA food monitoring data.

International Information.— Import commodities continue to represent an increasing proportion of the U.S. food supply. The Battelle World Agrochemical Data Bank, a computerized data base containing information on use of pesticides in foreign countries, has been used by FDA since 1986 to assist in the planning of FDA's monitoring of imported produce. In addition, the Pesticide Monitoring Improvements Act of 1988 requires that FDA enter into cooperative agreements with the governments of countries that are major sources of imported foods or whose imports into the United States may be important in terms of their volume, dietary significance, or potential pesticide problems.

Priority will be placed on obtaining information on pesticide usage from those countries that are not covered by existing data sources such as the Battelle data base. Countries will be asked to provide information identifying the pesticides used in the production, transportation, and storage of food products imported into the United States. If available, information on regional or local pesticide use, the individuals and agencies responsible for the registration and monitoring of the use of pesticides, the laboratories used by the country's government, and any documents that describe approved pesticide uses, application rates, and residue levels will be obtained. FDA has initiated negotiations with representatives of the various countries.

Total Diet Study

The Total Diet Study, also known as the Market Basket Study, is the other major approach used by FDA to monitor pesticide residues in the food supply. The Total Diet Study is designed to estimate the dietary intakes of pesticide residues (as well as industrial chemicals, toxic elements, and essential minerals) by 8 age/sex groups, from infants to senior citizens. Foods are purchased from local supermarkets or grocery stores by FDA personnel 4 (or sometimes 5) times per year throughout the United States. Each of the 4 market baskets represents a different geographic region and is a composite of foods collected in 3 cities in the particular region. The cities are changed each year. Each market basket contains 234 food items that have been chosen, based on nationwide dietary surveys, to represent the diet of the U.S. population. The foods are prepared table-ready—ranging from peeling bananas to making beef and vegetable stew—and then are analyzed for pesticide residues. These results, in combination with food consumption data, allow for calculation of the dietary intakes. This provides an estimate of the actual amount of pesticide residues consumed by the U.S. population.

RESULTS AND DISCUSSION

Regulatory Monitoring

Imports comprised over half of the total number of samples analyzed; foods from 89 countries were sampled.

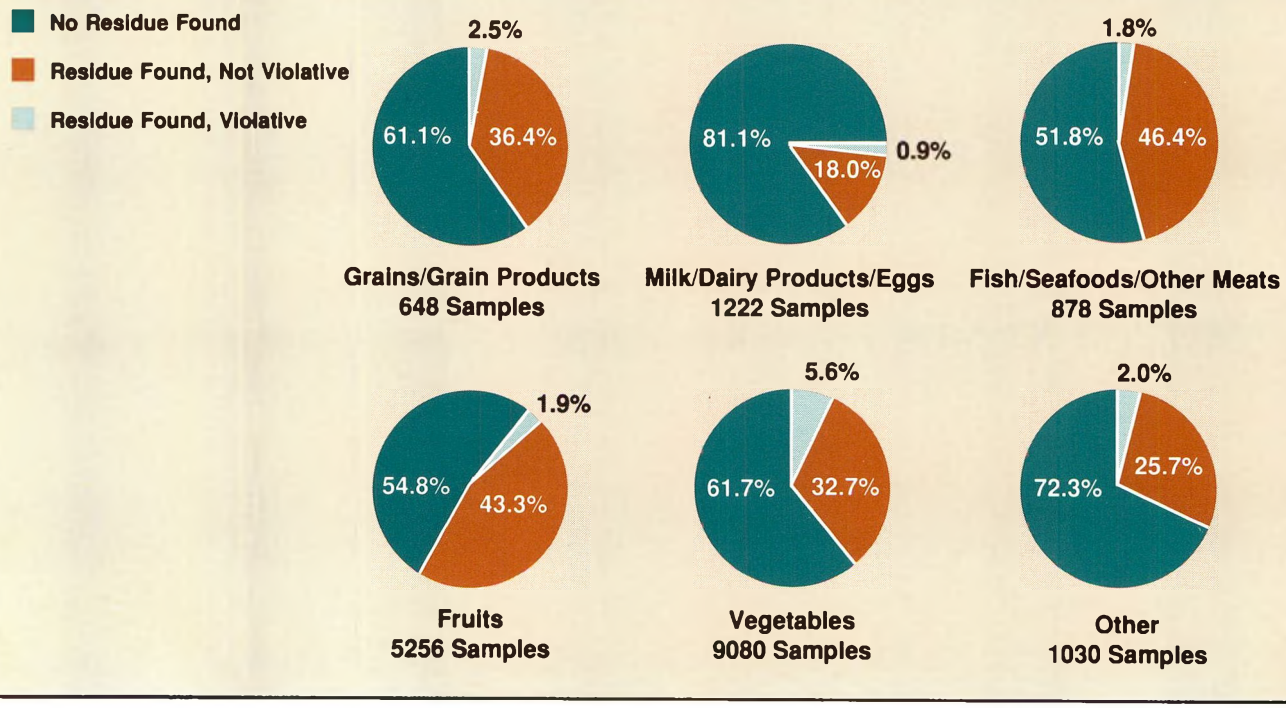
In 1988, 18,114 samples were analyzed through the regulatory monitoring approach, an increase of 25% over the total number of samples analyzed in 1987, reflecting a small increase in allocation of resources. Domestic and import sampling increased 17 and 31%, respectively, in 1988. Imported foods made up more than 57% of the total number of samples (55% in 1987), reflecting FDA's continuing emphasis on imports.

Figure 1 presents a summary of the residue findings (both domestic and import) for the 18,114 samples in terms of various commodity groups. More samples were analyzed in 1988 in every one of the food groups. As in 1987, fresh vegetables accounted for the largest number of foods sampled, followed by fruits and the milk/dairy products/eggs group. Overall, no violative residues were found in more than 96% of the samples, and in 61% of the samples, no residues were found. In 1987, no residues were found in 57% of the samples.

The lower limit of residue measurement in FDA's analysis for a given pesticide is well below the tolerance level. In general, residues present at 0.01 part per million (ppm) and above are measurable; however, for individual pesticides, this limit may vary from about 0.005 to 1 ppm. FDA attempts to maintain uniform application of the analytical methods in all of its laboratories.

Appendix A provides more detailed data by commodity group for domestic samples including the total number of samples analyzed, the percent samples with no residues found, and the percent violative samples. Of

Figure 1
Summary of Results (Domestic and Import) of 1988 Sample Analysis for Pesticide Residues by Commodity Groups



the 7639 domestic samples analyzed, 60% had no detectable residues, less than 1% had over-tolerance residues, and 1% had residues of pesticides for which there are no tolerances for the particular pesticide/commodity combination. No residues were found in the majority of samples reflecting foods of major dietary importance. For example, of the more than 3400 fresh vegetable samples analyzed, no residues were found in 65%, less than 1% contained residues over tolerance, and 2% contained residues for which no tolerance had been established. For fresh fruits, no residues were found in 49%, less than 1% contained over-tolerance residues, and less than 1% contained residues that had no established tolerance. No residues were found in 81% of the 946 domestic dairy product samples (milk, cheese, butter, etc.), and less than 1% contained violative residues.

Appendix B shows the findings for imported foods. Of the 10,475 import samples analyzed, 62% had no detectable residues, less than 1% had over-tolerance residues, and 5% had residues of pesticides for which there are no tolerances for the particular pesticide/commodity combination. Samples with residues of pesticides without tolerances were 6% for vegetables and 2% for fruits. Less than 1% of the fruit samples and less than 1% of the vegetable samples contained over-tolerance levels of residues. Included in the 3171 imported fruit samples that were analyzed were 133 samples of bananas and 27 samples of plantains (Appendix B). Of these, 81% of the bananas and 96% of the plantains had no detectable residues. No violative residues were found in any of these samples. In 1987, of 154 bananas and 17 plantains analyzed, 68 and 94%, respectively, had no detectable residues, and no samples were violative. For imported dairy products, 82% of the 276 samples had no detectable residues, no residues were over tolerance, and 2% contained residues that had no tolerance.

Geographical Coverage—Domestic.—During 1988, domestic samples were collected from all 50 states and Puerto Rico. Figure 2 shows the distribution of the sampling throughout the nation. The largest numbers of samples were collected from states where agriculture is a major industry.

Import.—Samples representing food shipments imported from 89 countries

Figure 2
Distribution of Sample Collection in 1988 from the United States and Puerto Rico

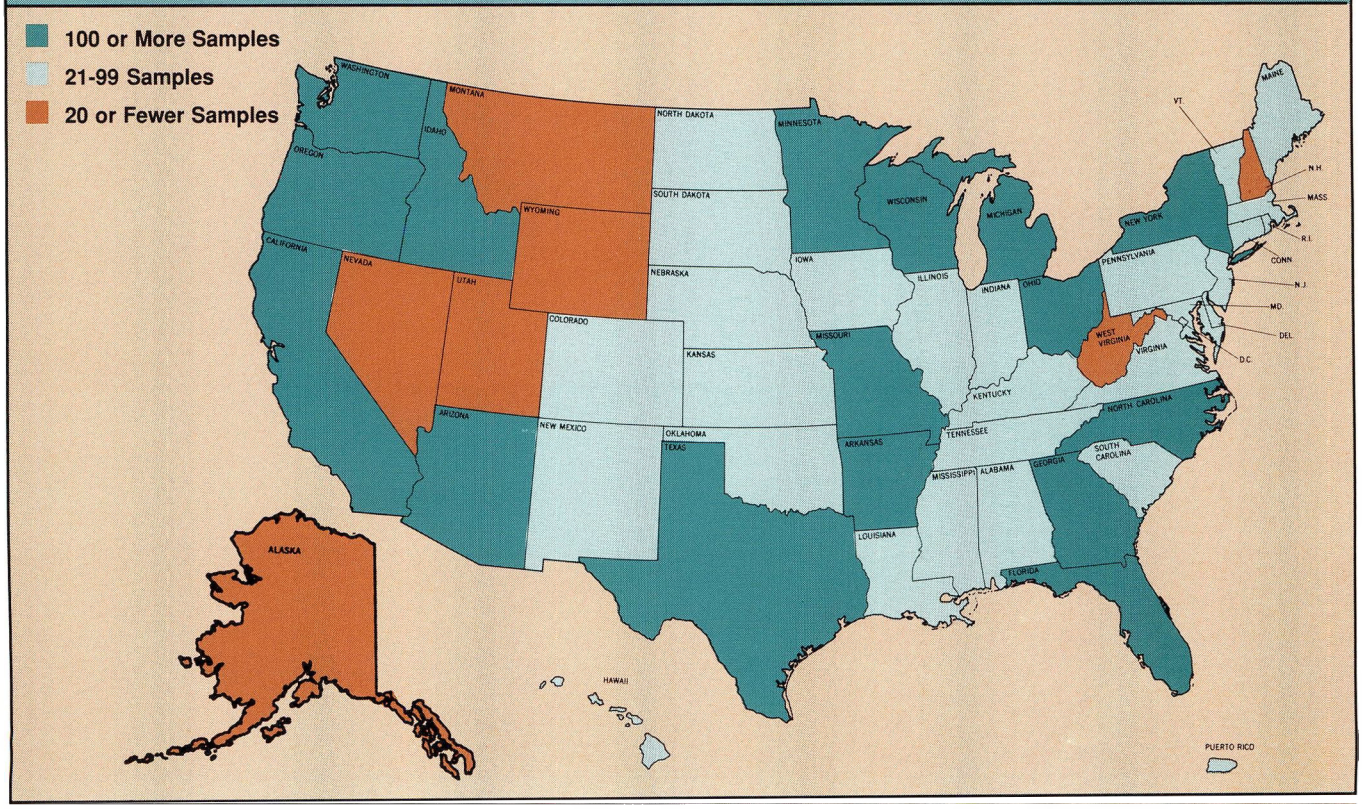


Figure 3
Distribution of Import Sample Collection in 1988

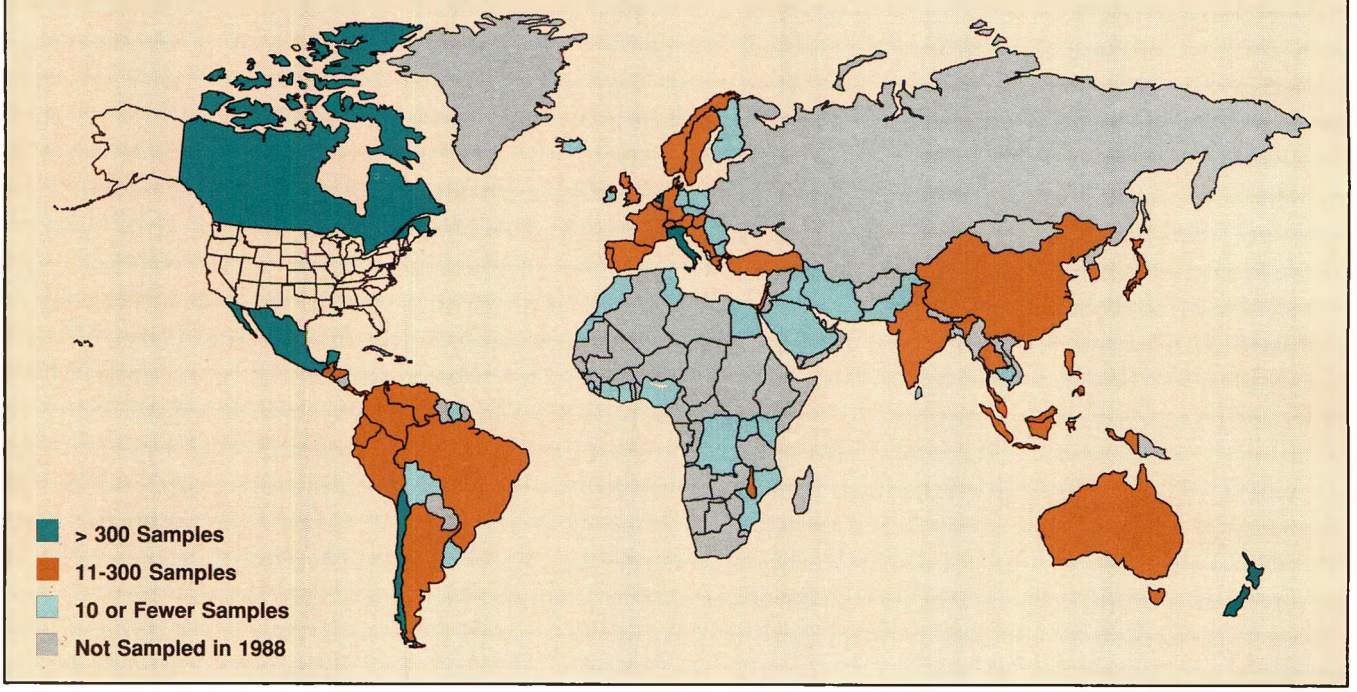


Table 1
Foreign Countries and Number of Samples Collected and Analyzed in 1988

Country	No. of Samples	Country	No. of Samples	Country	No. of Samples
Mexico	4385	India	62	Norway	11
Dominican Republic	901	Turkey	58	Switzerland	11
Chile	638	Brazil	54	Unspecified	26
Canada	501	Denmark	52		
New Zealand	352	United Kingdom	52	**Ten or fewer samples collected from the following:**	
Italy	326	Australia	51		
The Netherlands	304	Colombia	45	Bahamas	
Spain	274	Germany, Fed. Rep. of	38	Belize	
Thailand	255	Malaysia	32	Bolivia	
Guatemala	234	Haiti	30	Bulgaria	
China, Rep. of	213	El Salvador	27	Cambodia	
China, People's Rep. of	132	Portugal	27	Cyprus	
Costa Rica	104	Panama	25	Czechoslovakia	
Greece	101	Trinidad & Tobago	24	Egypt	
Jamaica	101	Venezuela	22	Finland	
Belgium	86	Indonesia	21	French Polynesia	
Honduras	86	Yugoslavia	21	Germany, Dem. Rep. of	
Argentina	81	Hungary	19	Ghana	
France	78	Peru	18	Grenada	
Israel	75	Lebanon	17	Guadeloupe	
Ecuador	74	Austria	15	Iceland	
Korea, Rep. of	73	Sweden	14	Iran	
Philippines	70	Malawi	13	Iraq	
Japan	69	Singapore	12	Ireland	
Hong Kong	62	Fiji Islands	11	Ivory Coast	
				Kenya	
				Macao	
				Morocco	
				Mozambique	
				Nigeria	
				Pakistan	
				Poland	
				Romania	
				Saudi Arabia	
				Sierra Leone	
				Spanish Sahara	
				Sri Lanka	
				Surinam	
				Tunisia	
				Uganda	
				Uruguay	
				Western Samoa	
				Zaire	

Table 2
Pesticides That Were Detectable by the Methods Used or Were Found in 1988 Regulatory Monitoring

Acephate*
Alachlor
Aldicarb*
Aldoxycarb*
Aldrin*
Allethrin
Ametryn
Amitraz
Amobam*
Anilazine*
Aramite
Aspon
Atrazine
Azinphos-ethyl
Azinphos-methyl*
Bendiocarb
Benfluralin
Benomyl*
Bensulide
BHC*
Binapacryl
Biphenyl
Bromacil
Bromophos
Bromophos-ethyl
Bromopropylate*
Bufencarb
Bulan
Butralin
Captafol*
Captan*
Carbaryl*
Carbendazim*
Carbofuran*
Carbon disulfide
Carbon tetrachloride*
Carbophenothion*
Carboxin*
Chlorbenside
Chlorbromuron
Chlordane*
Chlordecone
Chlordimeform
Chlorfenvinphos*
Chlornidine
Chlornitrofen
Chlorobenzilate*
Chloroform*
Chloroneb
Chloropicrin
Chloropropylate
Chlorothalonil*
Chloroxuron
Chlorpropham*
Chlorpyrifos*
Chlorpyrifos-methyl*
Chlorthion
Chlorthiophos
Clofentezine*
Clomazone
Coumaphos
Cyanazine
Cycloate
Cyhexatin*
Cypermethrin*
2,4-D*
2,4-DB
Daminozide*
DCPA*
DDT*
DEF*
Deltamethrin*
Demeton*
Dialifor
Diallate
Diazinon*
Dibromochloropropane
Dicapthon
Dichlobenil*
Dichlofenthion
Dichlofluanid*
Dichlone
Dichlorobenzene, ortho
Dichlorobenzene, para
1,3-Dichloropropene
Dichlorvos*
Diclofop-methyl
Dicloran*
Dicofol*
Dicrotophos*
Dieldrin*
Dilan
Dimethoate*
Dinitramine
Dinocap
Dioxacarb
Dioxathion
Diphenamid
Diphenylamine*
Disulfoton*
Diuron
Endosulfan*
Endrin*
EPN*
EPTC
Ethion*
Ethoprop
Ethoxyquin
Ethylene dibromide*
Ethylene dichloride*
Etrimfos
Famphur
Fenamiphos
Fenarimol
Fenbutatin oxide
Fenitrothion*
Fensulfothion
Fenthion*
Fenvalerate*
Fluchloralin
Flucythrinate
Fluometuron
Fluvalinate
Folpet*
Fonofos*
Gardona
Genite 923
Heptachlor*
Hexachlorobenzene*
Imazalil*
Iprodione*
Isobenzan
Isodrin
Isofenphos
Isoprocarb
Isopropalin
Jodfenphos
Leptophos
Lindane*
Linuron*
Malathion*
Mancozeb*
Maneb*
Mecarbam*
Mephosfolan
Merphos*
Metalaxyl
Metasystox thiol
Methamidophos*
Methidathion*
Methiocarb*
Methomyl*
Methoxychlor*
Methyl bromide
Methyl trithion
Methylene chloride
Metiram*
Metobromuron
Metolachlor
Metribuzin
Mevinphos*
Mirex
Monocrotophos*
Monolinuron
Nabam*
Naled
Napropamide
Nicotine
Nitralin
Nitrofen
Norea
Norflurazon
Omethoate*
Ovex
Oxadiazon
Oxamyl*
Oxydemeton-methyl*
Oxyfluorfen
Oxythioquinox*
Parathion*
Parathion-methyl*
Pendimethalin
Pentachlorophenol*
Permethrin*
Perthane*
Phenkapton
Phenthoate*
Phenylphenol, ortho*
Phorate*
Phosalone*
Phosmet*
Phosphamidon*
Phostex
Phoxim
Picloram
Pirimicarb*
Pirimiphos-ethyl*
Pirimiphos-methyl*
Procymidone*
Profenofos*
Profluralin
Prolan
Prometryn
Pronamide*
Propanil
Propargite*
Propazine
Propham
Propoxur
Prothiofos
Pyrazon
Pyrazophos*
Pyrethrins
Pyrethroids, synthetic*
Quinalphos*
Quintozene*
Ronnel
Salithion
Schradan
Simazine
Strobane
Sulfallate
Sulfotep
Sulfur*
Sulfur dioxide*
Sulprofos
2,4,5-T
2,3,6-TBA
TDE*
Tecnazene*
TEPP
Terbacil
Terbufos
Terbuthylazine
Tetradifon
Tetraiodoethylene
Tetrasul
Thiabendazole*
Thiodicarb
Thionazin
Thiophanate-methyl
Tolylfluanid
Toxaphene*
Triadimefon*
Triadimenol*
Triallate*
Triazophos
Tributyltin*
Trichlorfon*
Trichloronat
Trifluralin*
Trimethacarb
Vernolate
Vinclozolin*
Zineb*
Zytron

Some of these pesticides are no longer manufactured or registered for use in the United States.

* = pesticides that were found.

were collected. This is an increase of 10 countries over 1987, and represents the increasing emphasis on monitoring of imported foods. Figure 3 shows the distribution of the sampling by country, and Table 1 lists the number of samples collected and the countries from which they originated. As in 1987, the largest number of samples were collected from Mexican shipments, reflecting the volume and variety of commodities that are imported into the United States from that country, especially during the winter months.

Pesticide Coverage.—Table 2 lists the 256 pesticides that were detectable in commodities analyzed by the methods applied; the 118 pesticides that were found are also noted; these numbers are similar to those for 1987. As in the 1987 report, individually identified residues of isomers, metabolites, impurities, and alteration products have not been listed separately; only the "parent" pesticides from which they arise are named.

Commodity/Pesticide Residue Occurrence.—Several pesticides have been of particular interest recently, among them aldicarb, benomyl, and daminozide (trade name Alar). FDA's monitoring for these residues in the past few years indicates that, when found, their levels are well below the tolerances set by EPA.

Aldicarb.—In FY86 through FY88, 3737 domestic and import samples were analyzed by methods capable of determining aldicarb and its 2 major metabolites, the sulfoxide and the sulfone ("total aldicarb"). Of these, 3656 samples (98%) contained no detectable residues. Included in the 3737 samples were 312 potato samples (304 domestic and 8 import). No aldicarb residues were found in 82% of the samples. The highest level found was 0.71 ppm; the EPA tolerance on potatoes is 1 ppm. Also included in the 3737 samples were 55 samples of imported bananas. No residues of aldicarb were found in 98% of the banana samples. One sample contained 0.12 ppm total aldicarb (the tolerance is 0.3 ppm). Residues present at about 0.01 ppm and above would have been measured.

Benomyl.—In FY88, 352 samples of various foods (181 domestic and 171 import) were analyzed by methods capable of determining benomyl and related compounds. The samples included bananas; various berries; grapes; citrus, core, and pit fruits; pineapples; and several vegetables. Of the 352 samples, 31 domestic and 71 import samples contained detectable benomyl residues; no violative residues were found. Residues present at about 0.05 ppm and above would have been measured.

Daminozide.—In FY81 through FY88, 657 samples of various domestic and imported foods were analyzed for daminozide and/or UDMH (a daminozide impurity and degradation product). The majority of the samples were apples or apple products (dried, juice, juice concentrate, pomace, or sauce), but also included were canned and frozen sour cherries; cherry preserves; eggs; grapes; grape juice; grape syrup/topping; milk; peaches; peanuts; pears; peppers; squash; and tomatoes. When found, the residues were well below the tolerances for daminozide residues in these foods. The highest level of daminozide found in apples was 8.2 ppm. At the time (1981) the tolerance was 30 ppm; the present tolerance is 20 ppm. The tolerances for the other foods that were analyzed range from 0.02 ppm for milk, 0.2 ppm for eggs, and 0.5 ppm for tomatoes to 10–55 ppm for fruits and peanuts. Currently, residues present at about 0.2 ppm and above can be measured.

Selective Surveys.—A selective survey is designed to determine the occurrence of a particular pesticide or a group of chemically related pesticides in several different crops or to focus on a particular commodity or group of commodities. A number of selective surveys initiated by the FDA Center for Food Safety and Applied Nutrition were carried out for individual pesticides in 1988. Examples include surveys for aldicarb, benomyl and/or thiophanate-methyl, carbofuran, daminozide, and linuron. Surveys initiated or under way in 1988 that focused on food commodities included imported fresh and processed fruits and vegetables, foods imported from Africa, and imported processed stone fruits.

Automatic Detention.—Table 3 lists the commodities from specific shippers/countries that were subject to automatic detention in 1988. The list is more than double that for 1987 as a consequence of the new FDA procedure whereby automatic detention may be invoked when only *one* shipment is found to be violative. Requiring certificates of analysis, in effect, increases FDA's monitoring coverage.

Table 3
Automatic Detentions in 1988

Country	Commodity	No. of Shippers	No. of Pesticides
Belgium	corn salad	1	1
	leaf lettuce	1	1
Canada	cucumbers	1	1
	endive	1	1
Chile	asparagus	2	1
	pears	1	1
	raspberries	2	2
China, Rep. of	chinese pea pods	3	2
China, People's Rep. of	peppers	1	1
Colombia	lulos	1	2
Costa Rica	cantaloupe	1	1
	chayotes	1	2
	pumpkins	1	1
Dominican Republic	bitter melons	7	1
	cilantro	1	1
	eggplant	10	4
	green beans	2	1
	long beans	9	2
	okra	8	4
	peppers	9	2
	snow peas	3	5
	squash	15	5
	tamarinds	1	1
France	mushrooms	1	1
Guatemala	cantaloupe	1	1
	papayas	1	1
	snow peas	4	2
	squash	1	1
	sugar peas	1	1
Honduras	cantaloupe	1	1
	snow peas	1	1
Italy	endive	1	1
	grapes	1	1
Jamaica	calaloo	2	3
	peppers	3	3
	yams	1	1
Mexico	chayotes	1	1
	chinese beans	1	1
	coriander	all	5
	green beans	1	1
	kale	1	1
	lettuce	1	1
	peppers	20	7
	pineapples	1	1
	snow peas	1	1
	squash	3	3
	strawberries	2	2
	sugar peas	1	1
	tomatillos	12	4
Panama	honeydew melons	1	1
Spain	clementines	1	2
	lemons	5	2
The Netherlands	mushrooms	1	1
Venezuela	tomatoes	1	1

For simplicity, general commodities (including processed products) are listed rather than specific varieties.

No residues were found in over 60% of the foods sampled, and less than 1% contained residues that exceeded tolerances.

Surveillance/Compliance Violation Rate Comparison.—About 1% of the domestic and 4.2% of the import surveillance samples were violative (Figure 4). Since surveillance sampling is based on a number of factors including usage patterns and volume, commodity treatment history, dietary importance of the commodity, the toxicity of the pesticide, known problem areas, past monitoring results, and knowledge of local usage, the results may be biased toward higher violation rates than if the samples were collected in a truly random or statistical fashion. As expected, a much higher percentage of the compliance samples were violative: 24.9% for domestic and 14.5% for imports.

Summary: Regulatory Monitoring

In summary, no residues were found in over 60% of all samples (domestic and import) (Figure 5) analyzed under regulatory monitoring in 1988. Of those samples with violative residues, 84% of the violations occurred because there was no tolerance for that specific pesticide/commodity combination. Of the total number of samples, less than 1% had residue levels that exceeded tolerances. These data corroborate the findings and conclusions presented in the 1987 report, demonstrating again that pesticide residues in foods are generally well below tolerance levels.

Total Diet Study

Regulatory monitoring provides a general overview of the U.S. population's exposure to pesticide residues, whereas the Total Diet Study estimates pesticide residue intake via the diet for several age/sex groups. Analyses for the Total Diet Study are performed on foods prepared for consumption rather than on raw, unwashed commodities collected close to harvest, as is the case for regulatory monitoring. Thus, the Total Diet Study findings, in general, reflect the lower level of residues found in prepared food compared to raw commodities. In the Total Diet Study, 234 different food items representing the diets of U.S. consumers are collected. (In 1988, 5 market baskets were collected, resulting in a total of 1170 items.) Of the more than 200 chemicals that can be determined by the analytical methods used, 55 were found in the 1988 market basket foods. Because many residues dissipate with time and because food preparation may further reduce levels of pesticide residues, the analytical procedures used in the Total Diet Study are designed to permit measurement at levels 5 to 10 times lower than those employed in regulatory monitoring. If these lower levels were not attainable, far fewer residues would be found. Although the level varies with the chemical and food item, in general, residues present at or above about 0.001 ppm are measured. Residues consisting of a mixture of related chemicals generally are not measurable until a higher residue level is reached. The 30 most frequently found pesticides are listed in Table 4, together with the total number of findings and the percent occurrence in the 1170 samples. The 4 most frequently found pesticides were the same in 1988 and 1987.

Figure 4
Domestic and Import Surveillance Sample Violation Rates for 1988

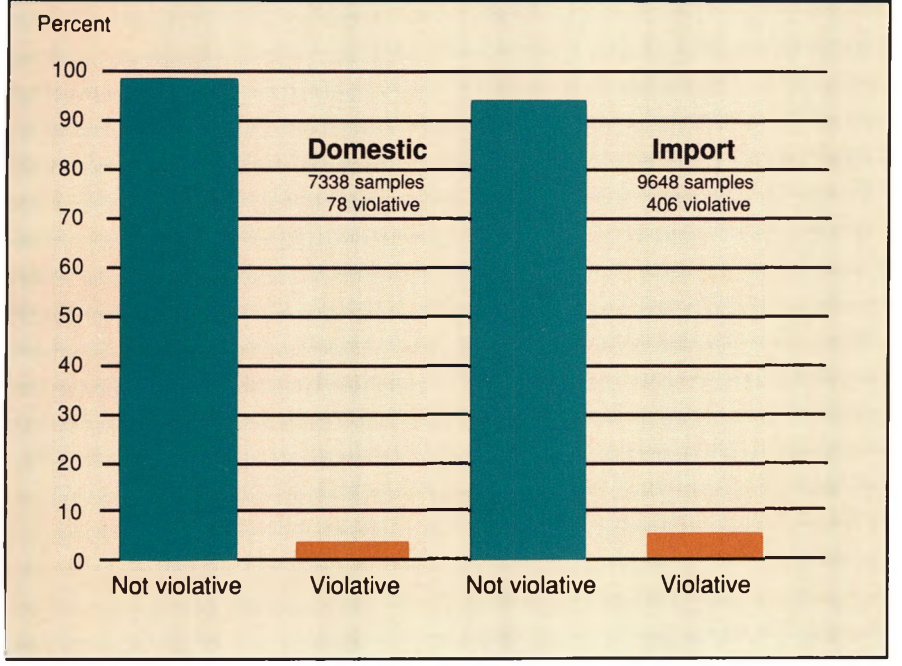
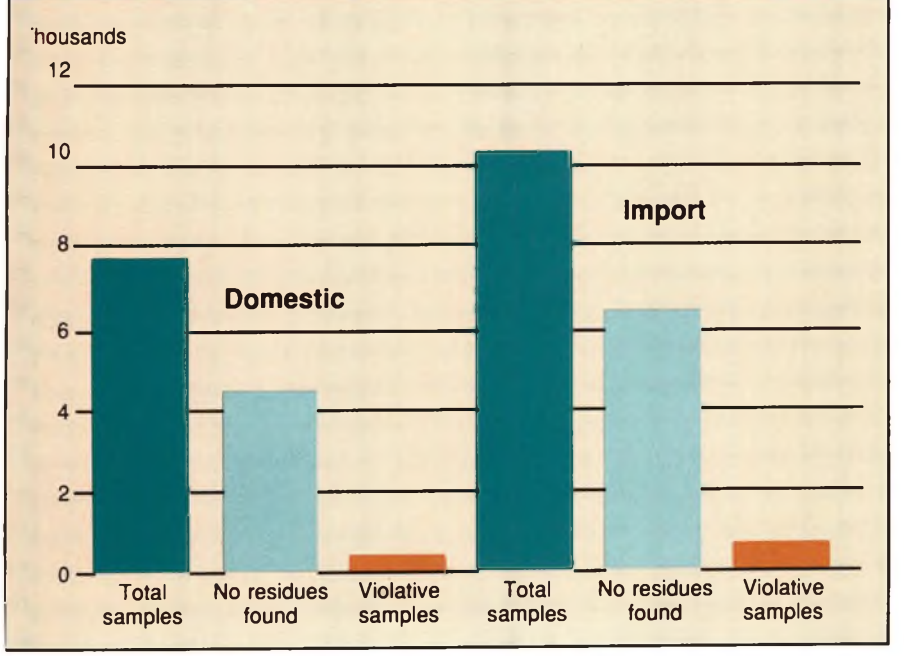


Figure 5
Comparison of Results for Domestic and Import Samples in 1988



Data collected over several years through the Total Diet Study can provide an historical perspective on pesticide residue occurrence in the food supply. For example, in the 6-year period from 1982 to 1988, 72 foods from each of 24 market baskets (1728 food items) were analyzed for aldicarb and its metabolites. Two samples were found to contain these residues: boiled potatoes (0.01 ppm) and fresh/frozen broccoli (0.02 ppm). During this same time, 24 banana samples were analyzed as part of the Total Diet Study. No pesticide residues were found in the edible portion of these samples. In 24 samples of banana and pineapple with tapioca (strained/junior foods), there was only one pesticide residue finding, malathion, at a level of 0.005 ppm.

ADI Comparisons.—The primary purpose of the Total Diet Study is to estimate the dietary intake of pesticides (plus industrial chemicals, toxic elements, radionuclides, and essential minerals) and compare these with acceptable daily intakes (ADIs). An ADI is the daily intake of a chemical which, if ingested over a lifetime, appears to be without appreciable risk. ADIs are established by scientific experts who attend annual joint meetings of the United Nations' Food and Agriculture Organization (FAO) and the World Health Organization (WHO) to evaluate, among other things, the toxicity of pesticides. Appendix C lists the intakes of a number of pesticide residues for 3 of the age/sex groups encompassed by the Total Diet Study and the ADIs for those pesticides. These are representative of the 8 age/sex groups typically covered. As in 1987, the dietary intakes of pesticide residues are below established ADIs. For most of the pesticides, these intakes are less than 1% of the ADI, reaffirming the wide difference between actual intakes in foods as consumed and estimated safe levels.

Summary: Total Diet Study

In summary, the findings of the Total Diet Study for 1988 corroborate the 1987 findings of very low levels of pesticide residues in food as consumed. As in 1987, the data show that pesticide intakes via the diet are generally manyfold lower than their ADIs. (Detailed information on the results of the Total Diet Study has been published in the *Journal of the Association of Official Analytical Chemists*.) The 1988 findings again show the continuing safety of the U.S. food supply relative to pesticide residues.

Table 4
Frequency of Occurrence of Pesticides in Total Diet Study in 1988

Pesticide[a]	Total No. of Findings[b]	Percent Occurrence	Pesticide[a]	Total No. of Findings[b]	Percent Occurrence
Malathion	242	21	Dicofol	27	2
DDT	237	20	DCPA	22	2
Diazinon	171	15	Parathion	20	2
Chlorpyrifos	124	11	Omethoate	19	2
Dieldrin	122	10	Phosalone	19	2
Chlorpyrifos-methyl	115	10	Toxaphene	19	2
Endosulfan	89	8	Permethrin	16	1
Hexachlorobenzene	86	7	Captan	13	1
Dicloran	76	6	Chlorobenzilate	12	1
Methamidophos	63	5	Sulfur	12	1
Heptachlor	58	5			
BHC, alpha & beta	52	4			
Lindane	48	4			
Chlordane	46	4			
Chlorpropham	40	3			
Dimethoate	39	3			
Quintozene	39	3			
Carbaryl[c]	35	3			
Acephate	33	3			
Ethion	29	2			

[a] Isomers, metabolites, and related compounds have not been listed separately; only the generic or "parent" pesticides from which they arise have been included.

[b] Based on 1170 items.

[c] Reflects overall incidence; however, only 72 selected foods were analyzed for *N*-methyl carbamates.

SUMMARY

A total of 18,114 samples of domestically produced food from all 50 states and Puerto Rico and imported food from 89 countries were analyzed for pesticide residues in 1988. This compares with 14,492 samples in 1987, with imports from 79 countries. No residues were found in over 60% of the samples, compared to 57% in 1987. Less than 1% of the 18,114 samples contained residues that exceeded the tolerances set by EPA. These findings, together with those from the Total Diet Study and the 1987 report, continue to demonstrate that dietary intakes of pesticide residues are well below standards set by FAO/WHO.

FDA pesticide monitoring concerned with imported foods was given increased emphasis in 1988. Automatic detention was invoked based on the finding of *one* violative sample of an imported commodity. A project was begun to obtain pesticide use data directly from a number of foreign countries that export foods to the United States.

This report confirms previous findings of very low levels of pesticide residues in the food supply.

This report was compiled through the efforts of the following FDA personnel: Sheila K. Egan, Kansas City District, Kansas City, MO; Ellis L. Gunderson, Marcia G. Houston, John W. Jones, Pasquale Lombardo, Donald V. Reed, Ronald R. Roy, and Norma J. Yess, Division of Contaminants Chemistry, Washington, DC; and John R. Wessel, Office of Regulatory Affairs, Rockville, MD.

Address reprint requests to: Norma J. Yess, Food and Drug Administration, Division of Contaminants Chemistry, HFF-420, 200 C Street, SW, Washington, DC 20204.

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APPENDIX A

Analysis of Domestic Samples[a] by Commodity Group in 1988

Commodity Group	Total No. of Samples	Percent Samples with No Residues Found	Percent Samples Violative: Over Tol.	Percent Samples Violative: No Tol.
A. Grains and Grain Products				
Corn, popcorn	74	47	1	0
Rice	73	82	1	1
Soybeans	42	57	0	0
Wheat	180	33	2	2
Other whole grains	26	42	0	4
Bakery & cereal products/snack foods	5	80	0	0
Rice products	18	100	0	0
Other grain products	18	61	0	0
Total	**436**	**51**	**1**	**1**
B. Milk/Dairy Products/Eggs				
Butter/butter products	28	61	0	0
Cheese/cheese products	124	73	2	0
Eggs/egg products	299	90	0	1
Milk & cream/milk products	495	79	0	0
Total	**946**	**81**	**<1**	**<1**
C. Fish/Seafoods				
Fish & shellfish **Total**	**447**	**28**	**2**	**1**
D. Fruits				
Blueberries	29	34	0	0
Cranberries	39	0	0	0
Grapes	149	72	0	0
Raspberries	33	73	0	0
Strawberries	257	24	<1[b]	<1
Other berries	24	58	0	0
Grapefruit	37	14	0	0
Oranges	160	37	0	0
Other citrus fruits	71	24	0	0
Apples	392	38	0	0
Pears	70	61	0	0
Other core fruits/ mixed fruits	2	0	0	0
Apricots	49	59	20	0
Cherries	101	50	1	0
Nectarines	49	51	0	0
Peaches	177	38	0	0
Plums & prunes	58	72	0	0
Other pit fruits	5	100	0	0
Papaya	22	100	0	0
Pineapples	31	100	0	0
Other tropical fruits	25	80	0	12
Cantaloupe	77	73	0	0
Honeydew	19	58	0	0
Watermelon	98	92	0	0
Other vine fruits	14	64	0	C
Other fruits	18	72	0	22
Fruit jams & toppings	16	69	0	C
Fruit juices	26	100	0	C
Fruits, dried or paste	37	86	0	C
Total	**2085**	**49**	**<1[b]**	**<1**
E. Vegetables				
Bean sprouts	11	100	0	0
Corn	123	98	0	0
Garden/green/sweet peas	62	92	0	
String beans	88	78	0	
Other beans, peas, & corn	30	70	0	1
Cucumbers	106	61	0	1
Eggplant	15	47	0	
Peppers	88	43	2	
Squash	76	86	0	
Tomatoes	225	75	0	<
Other fruits used as vegetables	6	83	0	
Artichokes	15	73	0	
Asparagus	43	98	0	
Broccoli	127	94	0	

Commodity Group	Total No. of Samples	Percent Samples with No Residues Found	Percent Samples Violative	
			Over Tol.	No Tol.
Brussels sprouts	19	95	0	0
Cabbage	140	90	0	0
Cauliflower	111	95	0	0
Celery	77	40	0	0
Chinese cabbage	64	55	0	31
Collards	36	39	0	8
Endive/chicory	70	61	7[b]	7
Kale	50	50	2	2
Lettuce	683	44	1	<1
Mustard greens	35	31	6[b]	6
Parsley	6	17	0	50
Spinach	118	54	0	3
Swiss chard	7	57	0	0
Turnip greens	37	19	3	3
Other leaf/stem vegetables	43	84	0	0
Mushroom/truffle products	32	44	0	0
Carrots	107	49	0	0
Leeks	11	91	0	0
Onions	99	76	0	1
Parsnips	9	22	0	33
Potatoes	323	71	2	0
Radishes	20	80	0	0
Red beets	15	67	0	0
Rutabagas	8	38	0	0
Sweet potatoes	122	68	0	3
Turnips	15	47	0	0
Other root/tuber vegetables	7	86	0	0
Vegetable juices	11	82	0	0
Vegetables, dried or paste	86	66	0	0
Vegetables with sauce	25	80	0	0
Total	**3401**	**65**	**<1[b]**	**2**
Other				
Spices & flavorings	20	95	0	0
Peanuts	34	65	0	0
Pecans	26	96	0	0
Sunflower seeds	6	100	0	0
Other nuts, seeds, & related products	15	87	0	0
Crude vegetable oil	45	80	0	0
Refined vegetable oil	30	73	0	0
Alcoholic beverages	40	40	0	0
Soft drinks & waters	15	100	0	0
Food sweeteners	23	70	0	22
Other food products	70	60	0	0
Total	**324**	**72**	**0**	**2**
A-F Total	**7639**	**60**	**<1[b]**	**1**

[a] Includes both surveillance and compliance samples.

[b] Includes samples that have both residue(s) over tolerance and residue(s) with no tolerance.

APPENDIX B

Analysis of Import Samples[a] by Commodity Group in 1988

Commodity Group	Total No. of Samples	Percent Samples with No Residues Found	Percent Samples Violative	
			Over Tol.	No Tol.
A. Grains and Grain Products				
Rice	64	95	0	0
Wheat	10	40	0	0
Other whole grains	11	82	0	0
Bakery & cereal products/snack foods	23	87	0	0
Pasta products	37	68	0	11
Rice products	41	98	0	0
Other grain products	26	58	0	4
Total	**212**	**82**	**0**	**2**
B. Dairy Products/Eggs				
Cheese/cheese products	210	89	0	0
Eggs/egg products	66	59	0	8
Total	**276**	**82**	**0**	**2**
C. Fish/Seafoods/Other Meats				
Fish & shellfish	418	77	0	0
Other meats	13	54	0	23
Total	**431**	**77**	**0**	**<1**
D. Fruits				
Blackberries	26	15	0	19
Blueberries	72	72	0	0
Boysenberries	10	20	0	0
Grapes	365	52	0	<1
Raspberries	105	42	1	3
Strawberries	169	17	0	0
Other berries	49	24	0	4
Grapefruit	12	83	0	0
Lemons	43	47	0	16
Limes	47	94	0	0
Oranges	120	54	0	2
Tangerines	32	22	0	3
Other citrus fruits	11	55	0	27
Apples	94	45	0	1
Pears	71	72	0	6
Mixed fruits	6	100	0	0
Apricots	24	29	0	0
Avocados	10	100	0	0
Cherries	31	39	0	0
Nectarines	47	4	0	0
Olives	124	91	0	2
Peaches	115	49	<1	2
Plums & prunes	53	19	0	0
Other pit fruits	13	92	0	0
Bananas	133	81	0	0
Breadfruit	11	100	0	0
Mangoes	79	99	0	0
Papaya	40	90	0	5
Pineapples	228	70	<1	2
Plantains	27	96	0	0
Other tropical fruits	120	81	0	8
Cantaloupe	309	38	<1	3
Honeydew	104	38	0	1
Watermelon	46	87	0	0
Bitter melons	105	78	0	10
Other vine fruits	34	68	0	3
Other fruits	10	20	0	0
Fruit jams & jellies	69	88	0	0
Fruit juices	81	79	0	0
Fruit toppings	27	93	0	4
Fruits, dried or paste	99	77	0	3
Total	**3171**	**58**	**<1**	**2**
E. Vegetables				
Blackeyed peas	13	77	0	0
Corn	42	100	0	0
Garbanzo beans/chick peas	23	78	4	0
Garden/green/sweet peas	347	49	3[b]	12
Mung beans	15	67	0	13
String beans	326	54	2	10
Other beans, peas, & corn	132	67	0	6
Cucumbers	256	37	<1	2
Eggplant	226	66	<1	4
Okra	159	74	0	9

ommodity Group	Total No. of Samples	Percent Samples with No Residues Found	Percent Samples Violative	
			Over Tol.	No Tol.
Peppers	1051	46	1[b]	7
Pumpkins	20	90	0	5
Squash	658	69	<1	6
Tomatillos	162	37	0	33
Tomatoes	364	45	<1	1
Other fruits used as vegetables	66	71	0	9
Asparagus	96	94	2	0
Bamboo sprouts	11	100	0	0
Broccoli	122	75	0	0
Broccoli raab	31	87	0	0
Brussels sprouts	51	71	0	2
Cabbage	37	89	0	0
Cauliflower	54	89	0	0
Celery	35	26	0	0
Chinese cabbage	36	33	0	25
Collards	14	21	0	0
Endive/chicory	93	77	0	4
Kale	41	15	0	10
Lettuce	143	50	1	2
Mustard greens	14	21	7	0
Parsley	37	16	0	49
Spinach	71	49	4	7
Turnip greens	25	12	16	0
Other leaf/stem vegetables	109	82	0	14
Mixed vegetables	8	50	0	0
Mushroom/truffle products	108	87	0	<1
Carrots	83	59	1	0
Leeks	15	93	0	0
Onions	85	79	0	0
Potatoes	30	77	0	0
Radishes	62	92	0	0
Red beets	27	70	0	0
Shallots	20	90	0	5
Sweet potatoes	21	90	0	0
Turnips	17	88	0	0
Water chestnuts	16	100	0	0
Other root/tuber vegetables	95	95	0	1

Commodity Group	Total No. of Samples	Percent Samples with No Residues Found	Percent Samples Violative	
			Over Tol.	No Tol.
Vegetables, dried or paste	160	71	0	6
Vegetables with sauce	45	64	0	0
Other vegetable-related products	7	100	0	0
Total	**5679**	**60**	**<1[b]**	**6**
F. Other				
Whole coriander	65	83	0	8
Other whole spices	83	81	0	6
Ground spices	21	48	5	5
Other spices & flavorings	11	91	0	0
Cashews	31	77	0	6
Coconuts	14	100	0	0
Other nuts & related products	25	92	0	0
Edible seeds & related products	18	78	0	6
Crude vegetable oil	75	51	0	0
Refined vegetable oil	170	65	0	0
Other vegetable oil products	11	91	0	0
Alcoholic beverages	16	94	0	0
Coffee & tea	45	73	0	2
Waters & nonalcoholic beverages	30	97	0	0
Chocolate & cocoa products	68	57	0	0
Other food products	23	96	0	0
Total	**706**	**73**	**<1**	**2**
A-F Total	**10475**	**62**	**<1[b]**	**5**

[a] Includes both surveillance and compliance samples.

[b] Includes samples that have both residue(s) over tolerance and residue(s) with no tolerance.

APPENDIX C

Pesticide Intakes (μg/kg body wt/day) Found in Total Diet Analyses and Their ADIs for 3 Age/Sex Groups in 1988

Pesticide	FAO/WHO ADI[a]	Age/Sex Group 6-11 mo.	14-16 yr M[b]	60-65 yr F[b]
Acephate	3	0.0105	0.0080	0.0104
Azinphos-methyl	2.5	0.0013	0.0039	0.0031
BHC, alpha + beta	--[c]	0.0008	0.0014	0.0010
BHC, gamma (lindane)	10	0.0008	0.0014	0.0009
Captan	100	0.0295	0.0141	0.0359
Carbaryl	10	0.0519	0.0086	0.0124
Chlordane, total	0.5[d]	0.0007	0.0007	0.0010
Chlorobenzilate	20	0.0055	0.0015	0.0011
Chlorpropham, total	--	0.1639	0.2140	0.0976
Chlorpyrifos	10	0.0141	0.0071	0.0061
Chlorpyrifos-methyl	10	0.0125	0.0175	0.0080
DCPA	--	0.0011	0.0009	0.0013
DDT, total	20[d]	0.0681	0.0264	0.0115
DEF	--	<0.0001	<0.0001	<0.0001
Diazinon	2	0.0057	0.0059	0.0033
Dicloran, total	30	0.1868	0.0394	0.0889
Dicofol, total	25	0.0217	0.0066	0.0156
Dieldrin	0.1[d]	0.0114	0.0049	0.0039
Dimethoate	10[d]	0.0077	0.0020	0.0063
Diphenylamine	20	0.0141	0.0417	0.0330
Endosulfan, total	8[c, e]	0.0376	0.0159	0.0224
Endrin	0.2	<0.0001	<0.0001	0.0001
Ethion	6[e]	0.0119	0.0039	0.0033
Fenitrothion	3	0.0014	0.0023	0.0017
Fenvalerate	20	0.0010	0.0006	0.0010
Folpet	10[e]	0.0009	0.0003	0.0011
Fonofos	--	0.0001	0.0001	<0.0001
Heptachlor, total	0.5[d]	0.0040	0.0017	0.0007
Hexachlorobenzene	--	0.0016	0.0011	0.0006
Iprodione, total	300	0.0017	0.0013	0.0014
Linuron	--	0.0025	0.0009	0.0012
Malathion	20	0.1360	0.1332	0.0819
Methamidophos	0.6	0.0126	0.0133	0.0253
Methidathion	5	0.0010	0.0009	0.0007
Methiocarb	1[d]	0.0001	<0.0001	<0.0001
Methomyl	10[e]	0.0026	0.0011	0.0028
Methoxychlor, *p,p'*	100	0.0003	0.0001	0.0001
Mevinphos, total	1.5[d]	0.0017	0.0008	0.0020
Monocrotophos	0.6	--[f]	<0.0001	0.0001
Omethoate	0.3	0.0024	0.0026	0.0062
Parathion, total	5	0.0073	0.0008	0.0015
Parathion-methyl	20	0.0002	0.0001	0.0002
Pentachlorophenol	--	0.0004	0.0002	0.0003
Permethrin, total	50[c]	0.0376	0.0737	0.1075
Perthane	--	0.0003	0.0001	0.0002
Phorate, total	0.2[d]	0.0001	0.0002	0.0002
Phosalone	6	0.1227	0.0007	0.0007
Phosmet	20[d]	0.0111	0.0032	0.0064
Pirimiphos-methyl	10	0.0002	0.0001	0.0001
Quintozene, total	7[c]	0.0009	0.0016	0.0008
Sulfur	--	0.0090	0.0119	0.0126
Tecnazene, total	10	0.0003	0.0002	0.0002
Toxaphene	--	0.0087	0.0078	0.0116
Triallate	--	0.0001	<0.0001	0.0001
Vinclozolin	40[d, e]	0.0071	0.0033	0.0081

[a] ADIs are usually expressed as mg/kg body wt/day but are expresse here as μg/kg body wt/day for ease of comparison.

The ADIs cited here reflect revisions made in 1987.

[b] M = male, F = female.

[c] ADI not established.

[d] Includes other (related) chemicals.

[e] "Temporary" ADI.

[f] No consumption of a food item containing this residue in this age group.

COLOR ADDITIVES

Determination of Citrus Red No. 2 in Orange Peel by Liquid Chromatography

CYNTHIA HOPE and RUSSELL CONNORS
T. J. Lipton, Inc., 800 Sylvan Ave, Englewood Cliffs, NJ 07632

A liquid chromatographic (LC) method is described for determination of the color additive Citrus Red No. 2 in orange peel. The dye is extracted with petroleum ether using a Soxhlet-type apparatus. The extract is evaporated almost to dryness and the residue is dissolved in acetonitrile. Liquid chromatographic analysis is carried out, isocratically, on an Ultrasphere ODS C18 column with acetonitrile–water (90 + 10) as mobile phase and detection at 504 nm. Recoveries from dried orange peel fortified at 3 levels ranged from 93 to 103%; recoveries from fresh orange peel ranged from 87 to 102%.

The color additive Citrus Red No. 2 is principally 1(-2,5-dimethoxy phenyl azo)-2-naphthol (Figure 1). It is often used to improve the color of the skins of whole oranges (1). The U.S. Food and Drug Administration regulations allow up to 2 ppm added color, based on the weight of fresh whole oranges in fruit which is not to be processed (2). No color should be added to orange peel intended for consumption, e.g., dried peel for flavoring herbal teas, candies, and cakes. Therefore, an analytical method which can detect low levels of the dye is needed.

The literature contains very few recent methods for determination of Citrus Red No. 2 in orange peel. In 1985, Love published a thin-layer chromatographic method for separating and identifying synthetic carotene derivatives, annatto, and Citrus Red No. 2 from natural carotenoids and xanthophylls (3). However, it was not quantitative. Ismail and Wardowski conducted a survey of Florida citrus packinghouse effluents for phenolic contaminants in 1974 (4). They concluded that the emulsion used for applying the dye, commonly known as color-add emulsion, contributes a very small percentage to the total phenolics in the effluents. However, the method they used for determination, the 4-aminoantipyrene colorimetric method in *Standard Methods of Analysis for Water and Wastewater* (5), is not specific and other phenolic contaminants such as pesticides and naturally occurring phenolics interfere.

Hayward published data on dye residues left on temple oranges after varying color-adding treatment conditions in 1962 (6). He found that residues were consistently less than the official tolerance of 2 ppm. The residues were determined by the method of Ting (7). In that procedure, the whole fruit was washed with 2 portions of chloroform and the absorbance of the resulting solution was measured with a spectrophotometer at 520 nm. The method was applicable only to dye on the surface of the fruit. Furthermore, there was no separation from naturally occurring pigments which also absorb at 520 nm.

In 1960, Ting and Deszyck (8) separated Citrus Red No. 2 and Red No. 32 on an aluminum oxide chromatographic column and determined them by spectrometry. They found 0.45–1.03 ppm Red No. 2 in 68 Hamlin oranges. However, the dye had been removed only from the surface of the oranges by rinsing in chloroform. In 1960, Przybylski et al. (9) separated chloroform washings of Citrus Red No. 2 and Oil Red XO (1-xylylazo-2-naphthol) by paper chromatography after preliminary purification on an alumina column. Dyes were extracted from the paper and determined quantitatively by spectrophotometry. However, no recovery data from orange samples were published by any of the above researchers, probably because of the difficulty in preparing spiked samples on whole oranges.

Other researchers have analyzed oil-soluble food colors by a variety of methods. Mark and McKeown (10) separated oil-soluble azo colors from foods by partitioning with dimethyl formamid and petroleum ether. Although they did not study Citrus Red directly, they found that azonaphthol colors, of which this dye is a type, were very stable and gave good recoveries. However, their method was time consuming, requiring many extractions and back extractions to obtain a residue pure enough to examine by spectrophotometry.

Paper chromatography was used by British researchers Pearson and co-workers to separate oil-soluble colors from light petroleum extracts (11). However, their method, while suitable for identification, was not sufficiently quantitative. The AOAC procedure for column chromatography of oil-soluble colors is time consuming, and some analysts have reported low recoveries (12).

METHOD

Apparatus

(a) *Extractor.*—Tecatur Soxhlet System Model 1043-005 (Tecatur, Inc., Herndon, VA).

(b) *Liquid Chromatograph.*—Beckman Model 110A (Beckman Instruments, Inc., Berkeley, CA 94710). Operating conditions; 20 μL loop injection valve; 1.5 mL/min flow rate.

(c) *LC Column.*—Ultrasphere ODS C18, 4.6 mm × 25 cm, 5 μm (Beckman Instruments, Inc.).

(d) *UV Detector.*—Spectroflow 757 (Kratos Analytical, Ramsey, NJ 07446), set at 504 nm wavelength.

(e) *Recorder.*—Kipp and Zonen Model BD 40 (Beckman, Instruments, Inc.); chart speed, 0.5 cm/min.

Reagents

(a) *Citrus Red No. 2.*—Certified (Buffalo Color Corp., Buffalo, NY).

(b) *Petroleum ether.*—Reagent grade.

(c) *Mobile phase.*—Reagent grade acetonitrile–water, (90 + 10). *Caution:* Acetonitrile is a possible carcinogen.

Extraction

Weigh 5.0 g dried orange peel or 8.0 g fresh peel into Soxhlet thimble. If sample is herbal tea blend, use 5.0 g for tea containing 20% or more peel and 10.0 g for tea containing less than 20% peel. Place glass wool plug over sample. Position in Soxhlet apparatus in fume hood and extract according to manufacturer's directions with 75 mL petroleum ether for 90 min if dried peel or herbal tea and 2.5 h if fresh peel. Evaporate on steam bath almost to dryness. Add 30 mL acetonitrile and warm on steam bath while swirling for 2 min. Quantitatively transfer to 50 mL volumetric flask and dilute to volume with acetonitrile. Filter aliquot through 0.45 μm Teflon filter.

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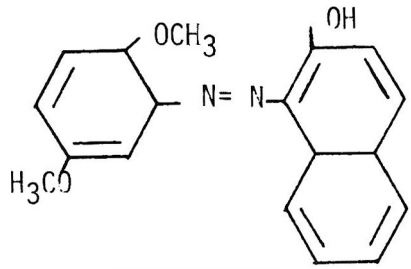

Figure 1. Structure of Citrus Red No. 2, 1(-2,5-dimethoxy phenyl azo)-2-naphthol.

Liquid Chromatography

Prepare 90/10 acetonitrile–water (90 + 10) mobile phase. Filter and degas. Equilibrate C18 column at flow rate of 1.5 mL/min. Set detector wavelength at 504 nm. Prepare standard solutions of Citrus Red No. 2. Weigh 0.1000 g into 100 mL volumetric flask and dilute to volume with acetonitrile for stock solution of 1000 μg/mL. Prepare serial dilutions with acetonitrile for standards of 2, 1, 0.8, 0.6, 0.4, 0.2 μg/mL. Filter through 0.45 μm Teflon filters. Inject standards and samples through 20 μL loop. Peaks will elute in approximately 4 min. Construct calibration curve by plotting peak height vs amount of standard. Calculate concentration of dye in solution by comparing peak height with calibration curve. Calculate concentration of dye in samples by multiplying by dilution volume and dividing by sample weight.

Results and Discussion

Liquid chromatography has provided a very sensitive means for the separation and quantitation of Citrus Red No. 2. The limit of detection in the extracts was 28 ppb. This was determined by a peak height at 3 times the noise level at 0.01 AUFS. Extraction of the dye from orange peel with the Soxhlet apparatus is simple and efficient, and requires little operator time. When applied to 6 herbal tea blends, dried orange peel from 3 suppliers, and fresh peel from a variety of sources, no interferences were found.

No Citrus Red No. 2 was found in any of the dried orange peel samples tested or in the herbal tea blends. Results of analyses of 14 fresh oranges randomly purchased in various supermarkets are given in Table 1. As can be seen, most were well below allowable limits. Four samples were above the limit. On 6 samples taken from one orange, the standard deviation was 0.06 μg/g on an average of 1.07 μg/g in the skin.

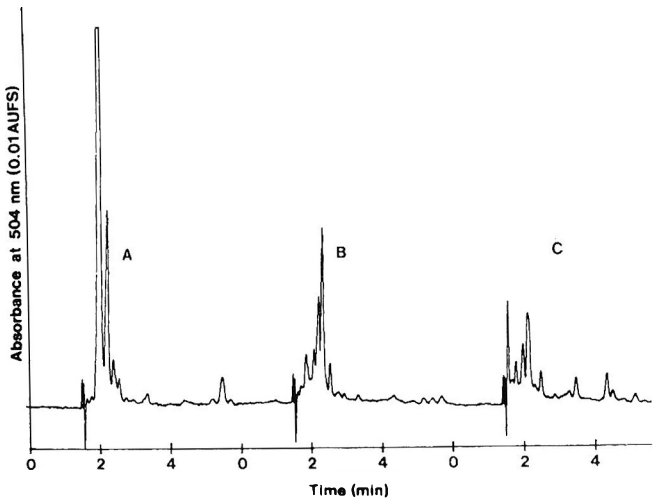

Figure 3. Chromatograms of extracts of 3 herbal tea blends containing A, 40%; B, 25%; C, 15% dried orange peel.

Shown in Figure 2 are chromatograms of extracts of dried orange peel from 2 different suppliers and of a sample fortified at 10 μg/g with Citrus Red No. 2, which elutes at 4 min. Sample B may have had a trace of Citrus Red No. 2, but the small peak at 4 min is too close to noise level to be quantitative.

Figure 3 shows chromatograms of extracts of three different herbal tea blends which contained (A) 40%, (B) 25%, (C) 15% dried orange peel.

Typical chromatograms of extracts of 2 types of fresh orange peel, which did not contain Citrus Red No. 2 are shown in Figure 4. Figure 5 shows a 250 ppb standard of Citrus Red No. 2 and an extract of fresh orange peel found to contain 2.0 μg/g of the dye.

Recovery studies were carried out on dried and fresh orange peel samples and one herbal tea blend fortified before the extraction stage. For the dried orange peel 2 mL, 4 mL, and 6 mL of 100 μg/mL standard solution, respectively, were added to 20 g samples to obtain 10, 20, and 30 μg/g fortifications. Samples were extracted after thorough drying in air. Results were 93.1, 97.6, and 103.9% at 10 μg/g; 94.1, 103.0, and 94.4% at 20 μg/g; and 99.7, 98.6, and 100.9% at 30 μg/g. Overall recovery averaged 98.4%.

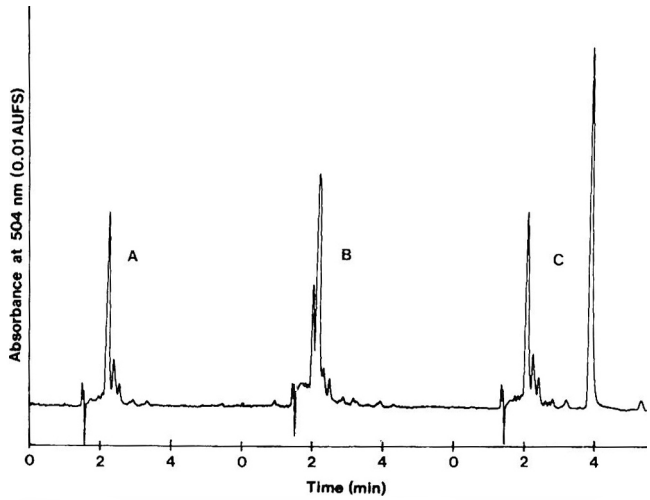

Figure 2. Chromatograms of extracts of dried orange peel from suppliers (A) and (B) and extract of (A) fortified at 10 μg/g.

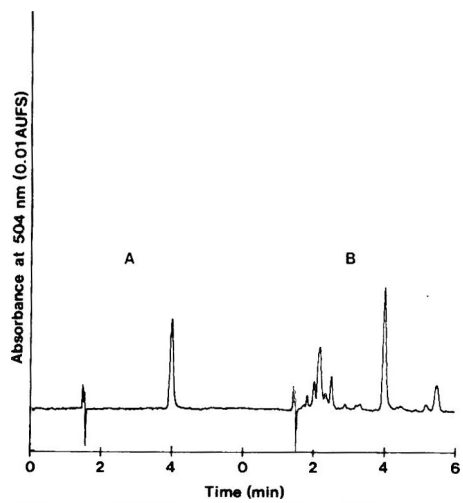

Figure 4. Chromatograms of extracts of fresh orange peel. A, Parson Brown and B, Hamlin.

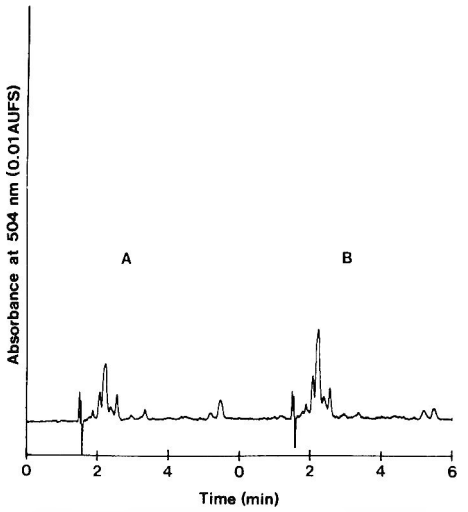

Figure 5. Chromatograms of A, 250 ppb standard of Citrus Red No. 2 and B, extract of fresh orange peel found to contain 2.0 μg/g of the dye.

For the fresh orange peel, 1 mL of standard solution was applied dropwise to the surface of pieces of pre-weighed peel while drying under a warm air stream. Thin skins could be fortified in one piece, and then folded and placed in extraction thimbles. Skins too thick to be folded easily were cut into smaller pieces before fortification. Recoveries were 93.9, 92.2, 87.3, 102.3, 94.3, and 90.1% for 10.4, 9.3, 12.3, 12.3, 19.2, and 18.7 μg/g added. Average recovery was 93.4%.

For the herbal tea, dried orange peel fortified as above at 10.68 ppm was used in preparing the blend. Concentration in the blend was 5 ppm. Recoveries on 3 samples of this were 97.2, 103, and 105%.

Further work on this project should include verification by another method, e.g., that of Przybylski et al. (9), recoveries from other herbal teas, and recoveries from lower level fortifications of dried orange peel.

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CHEMICAL JOURNALS ONLINE *Notes*

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COSMETICS

Rapid Bacteriological Screening of Cosmetic Raw Materials by Using Bioluminescence

PETER NIELSEN and ERIC VAN DELLEN
Amway Corp., Ada, MI 49355

Incoming cosmetic raw materials are routinely tested for microbial content. Standard plate count methods require up to 72 h. A rapid, sensitive, and inexpensive raw material screening method was developed that detects the presence of bacteria by means of ATP (bioluminescence). With a 24-h broth enrichment, the minimum bacterial ATP detection threshold of 1 cfu/g sample can be achieved using purified firefly luciferin-luciferase and an ATP releasing reagent. By using this rapid screen, microbiologically free material may be released for production within 24 h, while contaminated material undergoes further quantitative and identification testing. In order for a raw material to be validated for this method it must be evaluated for (*1*) a potential nonmicrobial light-contributing reaction resulting in a false positive or, (*2*) degradation of the ATP giving a false negative, and (*3*) confirmation that the raw material has not overwhelmed the buffering capacity of the enrichment broth. The key criteria for a rapid screen was the sensitivity to detect less than one colony forming unit per g product, the speed to do this within 24 h, and cost efficiency. Bioluminescence meets these criteria. With an enrichment step, it can detect less than one cfu/g sample. After the enrichment step, analysis time per sample is approximately 2 min and the cost for material and reagents is less than one dollar per sample.

All living cells contain ATP (adenosine triphosphate), a nucleotide essential to cellular metabolic pathways. When a microbial cell dies, ATP production ceases and the already formed ATP is rapidly destroyed by ATPase and the various phosphatases present. The ATP concentration contributed to a sample from live bacteria is measured by the enzyme-controlled bioluminescence reaction of luciferin-luciferase-ATP (Figure 1). To isolate ATP, a chemical releasing reagent is used to increase cell membrane permeability, which allows the release of ATP into the extracellular environment. Purified firefly luciferin-luciferase is then injected and reacts with ATP, to produce light (Monograph 202 (1981) Turner Designs). The light generated indicates the presence of live bacteria and is proportional to the amount of ATP present, which depends on the number of live organisms in the sample. The light given off in the reaction is detected by a luminometer, an instrument designed specifically to measure very low levels of light. The light measurement is reported in arbitrary units referred to as relative light units (RLUs). (See references (3-10) for background information.)

Experimental

Apparatus

(a) *Luminometer.*—Turner Designs Model 20e, including motorized auto-injector and printer system (Turner Designs, Mountainview, CA).

(b) *Centrifuge.*—Capable of holding 15 mL polypropylene test tubes.

(c) *Micro pipet.*—50 ± 0.1 μL Gilson Pipetman with Rainnin disposable pipet tips, 0-200 μL.

(d) *Petri dishes.*—Sterile, 15 × 100 mm, 2- and 4-section compartmented.

(e) *Vortex mixer.*

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Reagents

(a) *Luminescent grade luciferin-luciferase enzyme.*
(b) *ATP releasing reagent.*
(c) *HEPES buffer.*
(d) *ATP stock standard.*—Turner Designs, Mountainview, CA.
(e) *TAT broth base.*
(f) *MacConkey agar.*
(g) *Microbial content agar.*
(h) *Potato dextrose agar.*
(i) *Pseudomonas isolation agar.*
(j) *Columbia naladixic agar.*—Difco.

Preparation of Sample

Sample preparation consisted of weighing 10 g sample material into a wide-mouth dilution bottle containing 90 mL TAT broth. Bottles were then incubated 24 ± 2 h at 35° ± 2°C. After incubation, a 2 mL aliquot was removed from the dilution bottle, dispensed into a sterile 15 mL polypropylene centrifuge test tube, centrifuged 5 min at 1000 × *g*, and decanted. One mL sterile deionized water was added to the tube which was then vortex-mixed to resuspend the button of organisms. From the resuspended sample, a 50 μL aliquot was removed and placed in an 8 × 50 mm cuvet. To this was added 50 μL releasing reagent and 50 μL HEPES buffer, the tube was placed in the luminometer, and 100 μL luciferin-luciferase was dispensed by automatic injector.

Bioluminescence Detection

The amount of light emitted by bacterial ATP is displayed on an LED screen as RLUs (relative light units), an average of light output over an integration period of 10 s. A sample with 1 RLU value greater than the blank would indicate the presence of bacteria. The minimum sensitivity threshold of the instrument is approximately 10^3 to 10^4 organisms/mL, assuming 5×10^{-16} g ATP/cell (Monograph 204 (1983) Turner Designs). This is why a 24 h enrichment period is necessary. Under favorable conditions, a sample with 1 organism/g will attain this number within a 24 h period.

It should be noted that the 1 RLU value is only meaningful when it is related to a known concentration of ATP. This relationship should be established for each vial of luciferin-luciferase enzyme by running an ATP standard curve. These controls are essential because the enzyme activity is variable and decreases with time. The numeric output of the instrument should be corrected to account for these factors.

Confirmation

A sample with 1 RLU value greater than the blank was considered to be contaminated and was treated as follows: Using a sterile cotton-tipped applicator, the suspect dilution bottle containing the raw material was sampled and the sample was streaked onto a 15 × 100 mm bi-plate containing MacConkey agar and plate count agar. From these media, isolates were obtained for identification. In addition, the raw material was resampled and set up for quantitation according to CTFA guidelines for aerobic plate count (11).

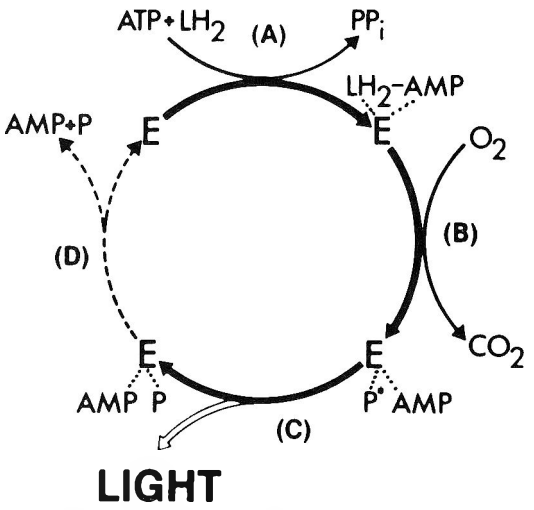

Figure 1. Firefly luciferase reaction.

A, In initial activating reaction, similar to activation of amino acids in protein biosyntheses, luciferin is adenylated by ATP to form luciferyl adenylate, bound to luciferase enzyme, and free pyrophosphate.

B, Luciferyl adenylate enzyme complex is oxidized by molecular O_2, resulting in CO_2, AMP, and electronically excited oxyluciferin. AMP and oxyluciferin remain bound to enzyme.

C, On returning to ground state, excited complex emits light. Color of light depends on firefly species (i.e., structure of the luciferase) and pH; it varies between greenish yellow (560 nm) and red (620 nm) (1). Under favorable conditions, quantum yields close to 100% have been observed.

D, In final step, luciferin and AMP are released from luciferase enzyme which is then free to enter another reaction cycle (2).

Table 1 lists some raw materials being evaluated by this procedure.

Results and Discussion

To thoroughly evaluate the application of this method for the bacteriological analysis of raw materials, the following criteria were investigated: (*1*) the selection of an enrichment broth; (*2*) the application of the method to the specific raw material; (*3*) challenge testing of each liquid raw material to determine its hostility toward microorganisms; and (*4*) a comparative analysis of the bioluminescence method with the standard aerobic plate count method as described by the CTFA Technical Guidelines, Test Product Sample Preparation.

Enrichment Broth Selection

Because the procedure depends on the growth of an organism within a 24 h period, 4 enrichment broths recommended for cosmetics were evaluated. The effectiveness of the 4 enrichment broths were compared for the individual growth response of each of the following organisms:

Pseudomonas aeruginosa ATCC 15442
Proteus vulgaris ATCC 6380
Citrobacter freundii
Staph. epidermidis ATCC 12228
Acinetobacter calcoaceticus
Pseudomonas stutzeri
Bacillus coagulans ATCC 8038

The broths were: Dey/Engly (D/E) (Difco); Letheen (Difco); *U.S. Pharmacopeia* Microbial Sterility Broth (TAT) (Difco); and a broth formula for the recovery of microorganisms from deionized water taken from Ref. (12). Ninety mL of each broth was dispensed into wide-mouth milk dilution bottles and then sterilized. A 24-h culture of the individual test organism in trypticase soy broth was diluted to yield 10–100 cfu/mL. This dilution was used to inoculate each broth. The inoculated broths were incubated in a water bath at 35 ± 2°C and a growth curve over an 8 h period was prepared.

There was no significant difference in the growth response among the 4 broths. Several raw materials are dry powders which will stress an organism. Combining that with rapid rehydration and nutrient shock by a broth, recovery of an organism could be severely limited. The criteria used to determine the broth of choice were formulation of the broth to avoid nutrient shock to the organisms; cost per liter; ease of making the broth; commercial availability of the broth. For these reasons, TAT broth was chosen for enrichment purposes.

The TAT broth was then evaluated for the ability to provide an optimum growth environment with the raw material. A raw material being strongly acidic or basic could shift the pH of the broth out of an ideal range of 6.8–7.2.

Ten g of a microbiologically clean raw material was weighed into milk dilution bottles containing 90 mL sterile TAT broth. With some raw materials such as gums, a reduced sample size of 1 g was used because more than 1 g would gel the enrichment broth (Table 2).

After the raw material had been dispersed in the enrichment broth, an aliquot was removed and the pH was checked. It was found that some raw materials exhausted the buffering capacity of the broth. Depending on the material, the pH would increase or decrease out of a favorable pH growth range for an enrichment broth (Table 3). Due to the pH shift of the broth, these raw materials are not acceptable for this

Table 1. Category of raw materials evaluated comparatively by luminescence and standard plate count

Raw material	Number evaluated	Raw material	Number evaluated
Emulsifiers	25	Abrasives	7
Surfactants	13	Humectants	8
Gums, thickeners	15	Emollients, moisturizers, conditioners	65
Dyes, pigments	41	Processing aids	2
Pearling agent	2	Resin	2

Table 2. Reduced sample size of gums, thickeners, and dyes

Raw material	pH	Sample size, g	Blank RLU	ATP std, ng	RLU with ATP std spike	Bacterial spike, CFU/mL	24 h RLU bacterial spike
Sodium stearate	9.33	1.0	0.613	60	383.8	0.71	111.0
Ultramarine Blue	7.30	1.0	0.262	60	184.5	0.46	3476
Silica	7.15	1.0	0.287	100	732	11	19.50
Hydroxyethyl cellulose	7.28	1.0	1.683	100	1322	11	69.5
						1.41	14.26
D&C Orange No. 5	5.97	1.0	0.210	100	0.122	1.45	9.24
FD&C Yellow No. 5	7.20	1.0	0.228	100	591.1	1.45	603.4

Table 3. Raw materials that shifted the pH of the enrichment broth

Raw material	pH	Sample size, g	Blank RLU	ATP std, ng	RLU with ATP std spike	Bacterial spike, CFU/mL	24 h RLU with bacterial spike
Cocamide MEA	8.50	10	0.824	60	405.2	0.71	0.840
Sodium stearate	9.33	1.0	0.613	60	383.8	0.71	111.0
Aluminum zirconium tetrachloro hydrey-gly	3.44	10	0.008	60	0.005	0.71	0.001
Sodium phosphate	4.88	10	0.005	100	0.4788	11	5.715
Lauramide DEA	9.25	10	0.138	100	309.9	3.0	0.222
Capramide	9.41	10	0.016	100	2.26	9.50	0.022
Isostearamidopropyl-morpholine lactate	4.74	10	0.185	100	354.6	9.50	0.339
Nonoxynol-9 methyl carboxylic acid	3.56	10	0.018	100	90.3	9.40	0.077

procedure and will require further work to determine if they could act as a microbial carrier. If this is the case, a customized broth or another method of analysis may be necessary.

Evaluating the Application of Bioluminescence for a Raw Material

Because the detection of microorganisms is based on the production of light caused by cleaving the ATP bond, it is essential to determine if there are any interferences which would either quench the light output, or enhance it. Of all the raw materials tested, only 4% were not applicable to the procedure due to a light quenching reaction of the raw material. None were found to contribute to the light output.

From a dilution bottle containing a sample of the sterile raw material in TAT broth, a 1 mL aliquot was removed. This aliquot was split into 2 portions, A and B. Portion A was assayed by the bioluminescence procedure to determine if the raw material contributed to the light output. Portion B was spiked with an ATP standard to determine any light quenching effect by the raw material. Essentially this helped determine the blank value for that raw material to be used as a criteria for bacterial contamination. A sample with 1 RLU value greater than the blank value for that raw material would be considered contaminated.

This same dilution bottle was then spiked with a dilute cocktail of organisms containing *Pseudomonas aeruginosa*, *Bacillus cereus*, *Proteus vulgaris*, and *Staph. epidermidis*. The total level of organisms ranged from 0.4 to 11/mL.

The spiked dilution bottles were then incubated at 35° ± 2°C for 24 ± 2 h. After incubation, the broth was checked qualitatively for the survival of the added organisms. The qualitative test was done by using a sterile cotton swab and swabbing the broth onto X plates, 4 quadrant petri dishes containing selective, differential, and a general media: *Pseudomonas* isolation agar, MacConkey agar, Columbia naladixic agar, and plate count agar. This provided a quick qualitative analysis of the surviving organism in each raw material. This would also be a check on the method to ensure against false negatives. The sample was also assayed according to the previously described method for ATP by bioluminescence.

Challenge Testing

Because liquid raw materials are more susceptible than powders to degradation by bacteria, liquid raw materials were challenged to determine if a liquid raw material was hostile to microorganisms, could act as a carrier, or would

Table 4. Example of raw materials being evaluated by bioluminescence

Raw material[a]	pH	Sample size, g	Blank RLU	ATP std, ng	RLU with ATP std spike	Bacterial spike, CFU/mL	24 h RLU with bacterial spike
Steareth-100	7.04	10	0.817	60	492.2	0.71	27.05
Hydrated Silica	6.88	10	0.904	60	616	0.46	41.81
Mica & Titanium & Chromium Oxide	6.86	10	0.428	60	308.8	0.46	9436
Isopropyl Lanolate	7.14	10	1.098	60	539.8	0.46	552.3
Glycerin	7.34	10	0.826	60	600	0.46	3838
PEG 40 Stearate	7.28	10	0.624	100	1210	1.45	31.83
Octocryzene	7.26	10	1.414	60	620.6	0.46	3651
Stearyl Alcohol	7.24	10	1.250	60	619.4	0.46	8411
Sesame Oil Mica (and) Bismuth	7.24	10	1.429	60	615.6	0.46	9999
Oxychloride, Bilite 20	7.20	10	0.834	60	137.4	0.46	3763
Iron Oxides	7.42	10	0.002	60	12.37	0.46	6451
Sorbitan Laurate	6.77	10	0.621	60	460.1	0.46	20.63
Tocopheryl Acetate	7.28	10	1.627	60	614.5	0.46	5092
Glycerin Natural	7.34	10	0.826	60	600	0.46	3838
Myristyl Lactate	7.22	10	1.968	60	595.0	0.46	2775
Dicalcium Phosphate	7.09	10	0.043	100	596.5	0.3	32.89

[a] CTFA nomenclature used.

Table 5. Comparison of bioluminescence and standard aerobic plate count methods

Time, h	Bioluminescence	Std aerobic plate count	
0	Sample/ enrichment broth	Sample/ enrichment broth	
			Quant. aerobic plate count bacteria/mold
24	Luminometer (RESULTS)	Sterility check	10/g bacteria/mold
48			Read quant. bact. aerobic plate count
77		Read bacterial plate	
96			Read quant. mold aerobic plate count
120		Read mold plate (RESULTS)	

promote growth. It was challenged by the following procedure:

A 100 g aliquot of liquid raw material was inoculated with 1 mL of 1.0×10^8/mL of a mixture of *Staph. epidermidis* ATCC 155, *Escherichia coli* ATCC 11229, *Proteus mirabilis* ATCC 9921, *Enterobacter cloacae* ATCC 7256, *Alcaligenes denetrificans* ATCC 15173, *Enetric streptococcus* ATCC 19432, *Pseudomonas aeruginosa* ATCC 15442, *Saccromyces* sp., *Aspergillus* sp., and *Penicillium* sp. Then, 10 g inoculated sample was removed at 0 h, 1 day, 2 days, and 4 days. This sample was dispensed into a dilution bottle containing 90 mL TAT broth, incubated 24 ± 2 h at 35° ± 2°C, and then streaked with sterile cotton tipped swab onto Petri dishes containing microbial content agar and potato dextrose agar. The microbial content plates were then incubated 24 ± 2 h at 35° ± 2°C and examined for visible growth. The potato dextrose agar was incubated 96 ± 2 h at 30° ± 2°C and examined for visible growth. Raw materials that self-sterilized before 4 days and did not contain a preservative system were considered hostile: lauramide DEA, capramide DEA, cocamidopropyl betaine, TEA-lauryl sulfate, and diethylaminoethyl stearate.

A nonhostile product as well as a preserved raw material could be screened microbiologically using the bioluminescence method. Hostile raw materials could be made exempt from future microbiological testing.

In-Use Comparison of Bioluminescence with Standard Plate Count Method

To further validate bioluminescence as a rapid screen for bacterial contamination of production raw materials, our Quality Assurance Department ran a comparative study with the bioluminescence method and the standard plate count method with incoming raw materials for 9 months. Table 4 lists general categories of samples tested. In this comparative study, using validated raw materials, the results indicating the presence or absence of bacteria obtained by standard plate count correlated perfectly with results from the luminescence method. The luminescence method provided results 3 days sooner than the standard plate count method. Table 5 illustrates a comparison of the bioluminescence method with the standard plate count method.

Conclusion

If a cosmetic raw material is evaluated as described and found to be applicable to analysis by bioluminescence, then that material may confidently be screened routinely by this method, resulting in signifcant savings in laboratory analysis time and raw material inventory and allowing expeditious release of finished product.

Acknowledgments

Special thanks to Jeff Boggs who coordinated the quality assurance effort, Barb Korthuis, and Karl Van Dyke.

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DAIRY PRODUCTS

Comparison of Two Methods for Determination of Total Solids Content of Milk: Collaborative Study

JENNY L. CLARK and DAVID M. BARBANO
Cornell University, Department of Food Science, Ithaca, NY 14853
CHAPMAN E. DUNHAM
Texas Milk Market, PO Box 110939, Carrollton, TX 75011

Collaborating laboratories: Cornell University; and laboratories operated by or under contract to the following Federal Milk Markets: Central Arizona; Chicago Regional; Eastern Ohio/Western Pennsylvania; Louisville-Lexington-Evansville; New England; Southern Illinois-Eastern Missouri; Southwest Plains; Texas; Upper Midwest

Ten laboratories analyzed 9 pairs of blind duplicate raw milk samples for total solids. A direct forced air oven method (4 h at 100°C) and a modification of the AOAC predry method (16.032) were used. Preliminary evaluation of the modified AOAC method indicated that blank determinations were necessary. Total solids content ranged from 12.0 to 14.6%. Average repeatability standard deviations (s_r) of the direct forced air oven and modified AOAC methods were 0.019 and 0.017, respectively. Average reproducibility standard deviations (s_R) of the direct forced air oven and the modified AOAC methods were 0.042 and 0.047, respectively. Average repeatability relative standard deviations (RSD_r) for the direct forced air oven and the modified AOAC methods were 0.149 and 0.136%, respectively; average reproducibility relative standard deviations (RSD_R) were 0.327 and 0.370%, respectively. Mean repeatability values (r) and reproducibility values (R) were 0.054 and 0.118 for the direct forced air oven method and 0.049 and 0.133 for the modified AOAC method, respectively. The mean test result of the direct forced air oven method (12.7293%) was comparable to that for the modified AOAC method (12.7273%). The modification of AOAC method 16.032 and the direct forced air oven method have been approved interim official first action.

Dairy farmers in some regions of the United States are paid on the basis of the total solids content of their raw milk, thus it is very important that an accurate reference method be used to measure total solids. Infrared milk analyzers can be calibrated to predict total solids (as in AOAC method **16.092** [1]) or solids-not-fat content of milks on the basis of absorbance of infrared light by fat, protein, and lactose. Analysis of milk samples for total solids content using an accurate reference method is necessary for proper calibration of infrared analyzers that estimate total solids or solids-not-fat.

Gravimetric methods involve weighing milk, drying milk, and weighing the dried milk residue. The total solids content of milk is the weight of the dried milk residue expressed as a percentage of the original milk weight. Maillard browning can occur during drying, which results in decomposition of solids components and gives a lower solids test value. Lactose can dry in the anhydrous and/or a stable α-monohydrate form (the monohydrate form increases the test result). Analytical conditions specified in gravimetric methods attempt to minimize browning reactions and to force lactose to dry in an anhydrous form.

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This report was presented at the 102nd AOAC Annual International Meeting, August 29–September 1, 1988, at Palm Beach, FL.
The recommendations have been approved interim official first action by the General Referee, the Committee on Foods I, and the Chairman of the Official Methods Board. The methods will be submitted for adoption official first action at the 103rd AOAC Annual International Meeting, September 25–28, 1989, at St. Louis, MO. Association actions will be published in "Changes in Official Methods of Analysis" (1990) *J. Assoc. Off. Anal. Chem.* 73, January/February issue.

Many researchers have attempted to modify and characterize various methods for determining the total solids content of milk samples (2–6). Boon (2) compared a Mojonnier method (2 mL milk predried on a hot plate for 2–3 min, then dried in a vacuum oven for 20 min); a British Standard method (2–3 g milk predried on a steam bath to dryness, then dried in an oven for 2.5 h at 100°C); and an aluminum cup method (1 mL milk dried 4.5 h at 102°C). The British Standard method is similar to AOAC method **16.032** (1). The British Standard method was determined to be the most reproducible and showed the least amount of browning. Green and Park (3) compared the AOAC method (12th Ed., **16.032**; 14th Ed., **16.032**) to a vacuum oven method. The AOAC method gave lower results (0.2%) than did the vacuum oven method, which was surmised to be caused by sample browning in the AOAC method.

A collaborative study (4) was conducted to obtain precision parameters (repeatability and reproducibility) for the International Dairy Federation (IDF) standard 21 A for determining total solids content of milk. The IDF method involves weighing 3 mL milk, predrying over a steam bath for 30 min, and drying in an oven at 102°C until the weight does not change (0.5 mg). The repeatability (r) and reproducibility (R) values were determined to be 0.10 and 0.16, respectively.

McDowell (5) compared the British Standard method, various direct oven heating methods (heating overnight, heating 4.5–5 h, and heating a smaller sample), and freeze-drying followed by Karl Fischer titration. He determined that the British Standard method gave an accurate assessment of total solids compared with Karl Fischer titration. Overall, direct oven heating for 4.5–5 h gave results similar to those for the British Standard method, although the differences between methods were larger for some samples than for others. Sherbon et al. (6) compared the AOAC method (11th Ed., **16.032**; 14th Ed., **16.032**) to a direct forced air oven drying procedure (4.5 h at 100°C), and found that the AOAC method yielded higher test values (0.035%).

The current AOAC method **(16.032)** involves predrying a milk sample over a steam table, and then drying it in an air oven for 3 h at 100°C (1). Gravimetric methods without steam table predrying are more direct (less chance of error), and are commonly used in the dairy industry. The objective of the present study was to compare the mean test value, mean repeatability, and mean reproducibility of a direct forced air oven drying method (no predrying) with the AOAC method (1).

Preliminary Work

Milks from 9 farms were selected for this study on the basis of a history of good milk quality (i.e., low bacteria counts and

low somatic cell counts). Farms were also selected to give typical variations in herd size and breed of cow. Seven of the farms were ≥98% Holstein herds. One farm was a 100% Jersey herd. One farm was 50% Holstein and 50% Brown Swiss. The mean herd size was 131 cows; the smallest farm consisted of 40 cows, and the largest of 300.

Ten laboratories located in different regions of the country participated in the study. Each laboratory conducted tests using both total solids methods. The same technician did not have to conduct both total solids procedures. Equipment currently used in total solids testing (balances and ovens) was evaluated, as were total solids dishes (size and manufacturer's processing treatment).

A preliminary comparison of the 2 methods was conducted in the Cornell University laboratory. The drying time was determined for the direct forced air oven method. It was also decided that blank values needed to be included in the calculations, particularly for the AOAC method. A complete description of each method and a set of practice samples were sent to all laboratories as indicated in the AOAC guidelines for carrying out a collaborative study (7).

Collaborative Study

Preliminary Work to Test Ruggedness of Methods

Two identical sets of 4 milk samples were sent to each of the participating laboratories for analysis by both methods (direct forced air oven drying and AOAC steam table predry method **16.032,** modified to include blanks). There were no blind duplicates; 1 set of samples was used per method. This served as a final check to confirm that both methods were being conducted properly. All laboratories participating in the study used Blue M ovens (Model 490A-2, or equivalent).

Collaborative Study Design

Before the collaborative study was begun, complete and detailed descriptions of the testing methods were developed on the basis of the ruggedness test. For the collaborative study, each laboratory received 2 different sets of 18 raw milk samples (9 pairs of blind duplicates in each set), a separate set of 18 samples for each method. Each pair of blind duplicates represented 1 farm. Blind duplicates were included within methods, between methods, and between laboratories. Sample testing order was random between methods and laboratories.

A 3-digit sample coding system was used. A computer program was prepared to collect data and to translate the codes to match blind duplicate test results, as well as to calculate AOAC statistical parameters and to conduct outlier tests (8, 9). Special data forms were sent to each laboratory to minimize the probability of sample mix-up. The data form for each set of 18 milk samples had all the sample numbers (3-digit code) printed on it in the order that the analyst was to test the samples. The forms also identified the method by which the analyst would test each set of 18 samples. In addition, a form for the analyst to write comments about each individual sample, and a questionnaire for each testing method, were included. The questionnaire was to be completed by the person conducting the testing. Information such as sample arrival time, start and finish time for each method, and actual testing conditions during the analysis were recorded on the questionnaire to help ensure that analysts followed the details of the procedures. Completed questionnaires were returned with the data sheets.

The week before the collaborative study, 180 six oz Whirl-Pak bags were coded with sample identification numbers, which were checked. Milks (5 L) were collected from 9 farms on a Monday morning (day 1) and transported on ice to the central laboratory. Milk samples were cold split in the central laboratory: the milk from each farm was mixed, poured into a large plastic container, and agitated continuously with a motor-driven stirrer while the milk was drawn from a spout (at the bottom of the container) directly into sample bags. Forty-six or more 6 oz Whirl-Pak bags of milk were prepared (80 mL per bag). Samples were refrigerated immediately after splitting. Sample splitting uniformity was verified immediately after each of the nine 5 L batches of milk was split by checking with a Milko-tester MK III **(16.068)** (1) the fat test of the first, middle, and last sample bag.

On Monday afternoon, when milk from all 9 farms had been split, samples were appropriately grouped, packed in ice, placed in insulated shipping containers, and sent by overnight air delivery to participating laboratories. On Tuesday (day 2), samples arrived at the laboratories and testing was initiated as soon as possible. All testing was completed by Friday (day 5). Test results and completed questionnaires arrived at the data analysis laboratory by the following Tuesday (day 9). All data were summarized and returned to individual testing laboratories by the next Wednesday (day 17). Collaborators verified that the data compiled for their laboratory were correct.

Verification of Milk Quality

A set of milk samples was tested for somatic cell count with a Foss-o-Matic electronic somatic cell counter (10), which was calibrated by direct microscopic somatic cell count (11). This set of samples had been exposed to all the same handling and shipping as the samples that were tested by both total solids methods.

Solids (Total) in Milk
By Forced Air Oven Drying After Steam Table Predry
Interim First Action

Method Performance:

$s_r = 0.017$; $s_R = 0.047$; $RSD_r = 0.136\%$; $RSD_R = 0.370\%$

A. Principle

Total solids are determined by weighing milk, drying milk, and weighing dried milk residue. Sample is predried 25 min on steam bath and then dried 3 h in 100 ± 1° forced air oven. Total solids content of milk is weight of dried milk residue as % of original milk weight.

B. Apparatus

(a) *Weighing dishes.*—Flat bottom, ≥5 cm diameter, without cover. If disposable aluminum dishes are used, they should be ones manufactured by "oil-free" process to allow more uniform spreading of sample in dish.

(b) *Calibration weights.*—Class S standard calibration weights to verify balance accuracy within weight ranges for weighing empty dish and dish plus sample.

(c) *Analytical balance.*—To read to nearest 0.0001 g. Accuracy on verification within 0.0002 g. Check periodically and whenever balance is moved or cleaned. Keep record of balance calibration checks. Digital electronic balance is recommended for fast response time for weighing warm milk.

(d) *Water bath.*—For tempering milk samples before weighing. Sample temperature should be 38 ± 1°.

(e) *Steam table.*—With individual openings that expose full bottom surface of each sample dish to steam.

(f) *Desiccator.*—Room temperature. For cooling weighing dishes after preliminary drying of empty dishes and after

Table 1. Percent total solids content of raw milk by direct forced air oven drying method

Material	Laboratory										Material mean
	A	B	C	D	E	F	G	H	I	J	
1	14.5339	14.5353	14.5001	14.6185	14.6449	14.5592	14.5509	14.6323	14.5749	14.6037	14.5834
	14.6250	14.5162	14.5198	14.5924	14.6727	14.5565	14.5457	14.5877	14.6595	14.6380	
2	12.4802	12.4630	12.4813	12.5234	12.5617	12.4819	12.4636	12.5366	12.5000	12.5103	12.5033
	12.4692	12.4697	12.4477	12.5028	12.5291	12.5660	12.4689	12.5803	12.5058	12.5249	
3	12.3778	12.3596	12.3836	12.3942	12.3900	12.4208	12.3608	12.4947	12.3956	12.4322	12.4050
	12.3510	12.3982	12.3659	12.4153	12.4272	12.4299	12.3597	12.5016	12.4112	12.4314	
4	12.0173	12.0031	12.0229	12.0197	12.0838	12.0513	11.9872	12.0991	12.0256	12.0738	12.0409
	12.0472	12.0216	12.0234	12.0477	12.0558	12.0692	11.9917	12.0797	12.0301	12.0678	
5	12.7901	12.7973	12.8037	12.8603	12.8464	12.8137	12.7853	12.8848	12.8529	12.8936	12.8341
	12.8431	12.8036	12.8138	12.8145	12.8382	12.8777	12.7976	12.8628	12.8565	12.8458	
6	12.2910	12.2803	12.3090	12.3477	12.3648	12.3389	12.2809	12.3954	12.3335	12.3391	12.3251
	12.2787	12.2898	12.2987	12.3173	12.3382	12.3452	12.2709	12.3975	12.3234	12.3622	
7	12.5848	12.6076	12.6150	12.6608	12.6543	12.6398	12.5938	12.7170	12.6485	12.6916	12.6501
	12.6018	12.6183	12.6390	12.6451	12.7195	12.6483	12.5992	12.8059	12.6380	12.6742	
8	12.8535	12.8291	12.8701	12.8930	12.9011	12.9026	12.8628	12.9912	12.9161	12.9023	12.8950
	12.8957	12.8333	12.8822	12.8947	12.9336	12.8932	12.8180	12.9914	12.9088	12.9274	
9	12.2775	12.2947	12.3058	12.3480	12.3510	12.3454	12.2870	12.4063	12.3476	12.3302	12.3267
	12.2786	12.2932	12.3053	12.3413	12.3444	12.3208	12.2831	12.4044	12.3292	12.3411	

final drying of dish plus sample. Use coarse desiccant (6–16 mesh) that contains minimum of fine particles and that changes color when moisture is absorbed. Desiccator should seal tightly and contain a thermometer.

(g) *Tongs.*

(h) *Forced air oven.*—Capable of rapid temperature recovery after sample loading and of maintaining 100 ± 1°. To evaluate temperature recovery, heat empty oven to 100 ± 1° and stabilize for 15 min. Turn oven off and fully open door for 2 min. Close oven door, turn oven back on, start timing, and record time for oven to reach 99°. Time should be ≤7 min.

C. Determination

(a) *Weighing dish preparation.*—Number clean weighing dishes and dry ≥2 h in 100 ± 1° forced air oven. Store dishes in desiccator until needed (must be used within 1 week or redried). Only touch predried dishes with clean, dry tongs.

(b) *Sample preparation.*—Temper milk to 38 ± 1° and mix until homogeneous as in **16.020.**

(c) *Initial weighing.*—(*Note:* Sample and weigh milk at 38 ± 1°. Record all weights to 4 decimal places.) Remove metal shelf from oven and place next to balance. Weigh predried sample dish. Pipet ca 3 g 38 ± 1° prepared milk sample (nearest 0.1 mg) directly into preweighed dish. Do not chase drift. Record sample weight immediately after weight stabilizes and just before start of steady drift to lower numbers. Remove dish from balance and place on oven shelf for transport to steam bath. Check balance zero between samples.

(d) *Blanks.*—Weigh 2 empty, predried dishes.

(e) *Predrying.*—Place sample dishes on steam bath so that full surface of dish bottom is exposed to steam. Be sure that all holes in steam bath contain a dish or are covered during predry period. Expose blank dishes to steam as for dishes containing samples. Do not have steam so high that dishes float or tip over. Predry milk until there is little or no free liquid movement in dish (≤25 min).

(f) *Drying.*—Preheat oven to 100 ± 1°. After steam table predry, place all dishes (including blanks) on oven shelf, turn oven off, and place shelf plus all dishes into forced air oven. (*Note:* Do not place dishes on solid trays; this does not allow heat to circulate evenly around dishes. Do not place anything else in oven.) Immediately after loading all samples in oven, close door and turn oven on again, and dry 3 h at 100 ± 1°. Do not open oven door until 3 h drying is completed.

(g) *Cooling.*—Remove dishes from oven and cool to room temperature in desiccator (≥30 min). Weigh dish plus dry milk on same balance that was used for initial weighings.

D. Calculation

Always use blank values in calculation. If mean blank is negative, be sure to add blank instead of subtracting it. Cause of negative blanks should be determined and corrected. Negative blanks are usually caused by pans not being completely dry before use in analysis.

$$\text{Total solids, \%} = \{[(W_2 - W) - B]/(W_1 - W)\} \times 100$$

where W = weight of dish; W_1 = weight of dish + milk sample; W_2 = weight of dish + dry milk; and B = mean blank weight.

Maximum recommended difference between duplicates = 0.05% total solids.

Ref.: JAOAC **72,** September/October issue (1989).

Solids (Total) in Milk
By Direct Forced Air Oven Drying
Interim First Action

Method Performance:

$s_r = 0.019$; $s_R = 0.042$; $RSD_r = 0.149\%$; $RSD_R = 0.327\%$

A. Principle

Total solids are determined by weighing milk, drying milk, and weighing dried milk residue. Sample is dried 4 h in 100 ± 1° forced air oven. Total solids content of milk is weight of dried milk residue expressed as % of original milk weight.

B. Apparatus

See predry method, **B (a)–(d)** and **(f)–(h).**

C. Determination

See predry method, **C (a)** and **(b),** and in addition:

(a) *Initial weighing.*—(*Note:* Sample and weigh milk at 38 ± 1°. Record all weights to 4 decimal places.) Remove metal shelf from oven and place next to balance. Weigh predried sample dish. Pipet ca 3 g 38 ± 1° prepared milk

Table 2. Percent total solids content of raw milk by steam table predry method

Material	Laboratory										Material mean
	A	B	C	D	E	F	G	H	I	J	
1	14.6253	14.5069	14.5318	14.5449	14.6362	14.5261	14.5386	14.6674	14.5682	14.6167	14.5827
	14.6059	14.5338	14.5621	14.5781	14.5917	14.5692	14.5591	14.6725	14.6020	14.6178	
2	12.5332	12.4513	12.4830	12.4768	12.5362	12.4697	12.4764	12.5893	12.5062	12.5355	12.5039
	12.5499	12.4630	12.4415	12.4337	12.5388	12.4859	12.4562	12.5970	12.5117	12.5427	
3	12.3685	12.3796	12.3796	12.3261	12.4393	12.4039	12.3549	12.4895	12.3974	12.4593	12.3964
	12.3745	12.3907	12.3146	12.3430	12.4007	12.4124	12.3350	12.5048	12.4040	12.4502	
4	12.0420[a]	12.0097	12.0110	11.9994	12.0716	12.0136	11.9996	12.1101	12.0616	12.1086	12.0377
	12.6979[a]	12.0091	12.0059	11.9873	12.0726	11.9993	12.0080	12.0790	12.0469	12.0850	
5	12.8308[a]	12.7874	12.8281	12.8231	12.8241	12.8042	12.8027	12.8748	12.8260	12.8861	12.8283
	12.3719[a]	12.8079	12.8120	12.8005	12.8409	12.7904	12.7826	12.9224	12.8454	12.8511	
6	12.3626	12.2689	12.2905	12.2783	12.3402	12.3188	12.2607	12.4331	12.3228	12.3838	12.3263
	12.3457	12.2942	12.2743	12.2861	12.3319	12.3043	12.2986	12.4283	12.3224	12.3802	
7	12.0349[a]	12.6315	12.6063	12.6338	12.6791	12.6421	12.6168	12.7635	12.6768	12.7018	12.6574
	12.6541[a]	12.6109	12.6325	12.6189	12.6620	12.6367	12.6314	12.7446	12.6499	12.6955	
8	12.8953	12.8467	12.8865	12.9273	12.9100	12.8654	12.8635	13.0167	12.9065	12.8976	12.8961
	12.8522	12.8473	12.8384	12.8451	12.9145	12.8943	12.8731	12.9737	12.9144	12.9537	
9	12.8514[a]	12.2783	12.2736	12.2746	12.3396	12.2911	12.2868	12.4045	12.3285	12.3245	12.3171
	12.2870[a]	12.2996	12.2743	12.2857	12.3191	12.3206	12.2699	12.4276	12.3261	12.3830	

[a] Cochran and Grubbs statistical outlier; not included in material mean.

sample (nearest 0.1 mg) directly into preweighed sample dish. Do not chase drift. Record sample weight immediately after weight stabilizes and just before start of drift to lower numbers. Remove dish from balance and place on oven shelf for transport to oven. Check balance zero between samples.

(b) *Blanks.*—Weigh 2 empty, predried dishes.

(c) *Drying.*—Preheat oven to 100 ± 1°. Open hot air exhaust vent to let moisture be removed from oven. After samples are weighed, turn off oven and place shelf plus all dishes (including blanks) into forced air oven. (*Note:* Do not place dishes on solid trays; this does not allow heat to circulate evenly around dishes. Do not place anything else in oven). Immediately after loading all samples in oven, close door and turn oven on again, and dry for 4 h at 100 ± 1°. Do not open oven door until 4 h drying is completed.

(d) *Cooling.*—Remove dishes from oven and let cool to room temperature in desiccator (≥30 min). Weigh dish plus dry milk on same balance that was used for initial weighings.

D. Calculation

See predry method, **D.**

Ref.: JAOAC **72,** September/October issue (1989).

Results and Discussion

Preliminary Work

One laboratory evaluated samples of dishes from all the participating laboratories. When evaluating total solids dishes in the preliminary work, we discovered that some disposable aluminum dishes were treated with an agent that prevented milk from spreading evenly across the bottom of the dish, while other dishes allowed the milk to spread uniformly over the bottom of the dish, so as to expose a greater surface area for evaporation. Both types of aluminum dishes were used in this study. Influence of dish on test results was measurable. Some dishes gained weight when exposed to steam (as much as 0.0020 g); others did not. The bottom of dishes from some laboratories developed a white film on the surface (when exposed to steam), which resulted in a weight gain. This may have been due to oxidation of either the aluminum or the coating on the aluminum. To account for this weight gain, blanks were used in both methods.

Due to sample evaporation and convection inside the balance, it is difficult to weigh raw milk samples at 38°C. Samples in this study were warmed and then kept warm to allow uniform dispersion of fat in solution and to minimize adhesion of fat to the sample container. Digital electronic balances allow the analyst to weigh a sample faster than on mechanical balances. Digital electronic balances were not specified for this research, however, that type was used by most collaborators in this study. Because they allow faster weighing, digital electronic balances are recommended for weighing warm samples.

Two types of ovens are commonly used in total solids drying: forced air ovens and gravity convection ovens. Vacuum ovens have been used, but have very poor temperature uniformity (6). Gravity convection ovens were not deemed appropriate for the study; by their nature, they have a slower recovery time after the oven is opened (e.g., when samples are placed in the oven) and a slower rate of removal of moist air from the oven. Therefore, because of their better temperature uniformity, more efficient moisture removal, and faster temperature recovery after opening, this study utilized only forced air ovens.

The ability of ovens to return to the testing temperature rapidly after being open and closed was evaluated by the following procedure: Heat empty oven to 100 ± 1°C and stabilize for 15 min, turn off oven and fully open door for 2 min, close oven door, turn oven back on (time zero), and record time that it takes oven to reach 99°C. All forced air ovens used in this study were capable of returning to 99°C in ≤7 min.

Preliminary Collaborative Study

At the time of the preliminary collaborative study (using 4 samples), one laboratory was not prepared to conduct the total solids determination by the steam table predry method. The results reported here for each method are from 9 laboratories. The overall mean test results were 13.2359% for the total solids direct forced air oven method, and 13.2478% for the modified AOAC method; the mean difference was 0.0119% total solids. Blank values were higher and more variable for the steam table predrying method than for the direct forced air oven drying method. Thus, we decided to use

Table 3. Comparison of mean test results of direct forced air oven method and steam table predry method by material for determination of total solids in raw milk

Material	Mean % total solids: Direct forced air oven, method 1	Mean % total solids: With steam table predry, method 2	% Total solids, difference method 1 minus method 2
1	14.5834	14.5827	0.0007
2	12.5033	12.5039	−0.0006
3	12.4050	12.3964	0.0086
4	12.0409	12.0377	0.0032
5	12.8341	12.8283	0.0058
6	12.3251	12.3263	−0.0012
7	12.6501	12.6574	−0.0073
8	12.8950	12.8961	−0.0011
9	12.3267	12.3171	0.0096
Mean	12.7293	12.7273	0.0020

Table 5. Mean blank values for determination of total solids in raw milk

Lab.	Mean blank, g: Direct oven	Mean blank, g: Predry
A	0.0001	0.0002
B	−0.0003	0.0001
C	0.0001	0.0022
D	0.0001	0.0025
E	0.0001	0.0004
F	−0.0001	0.0011
G	0.0001	0.0011
H	−0.0003	0.0010
I	0.0001	0.0004
J	0.0007	0.0024
Mean	0.0000	0.0011
Maximum	0.0007	0.0025
Minimum	−0.0003	0.0001
Range	0.0010	0.0024

the blank values in the calculations for the full collaborative comparison study. No laboratories reported problems with the methods. After conclusion of the preliminary collaborative study, 9 laboratories were deemed ready for the full collaborative study. The tenth laboratory, which had not had a steam table, obtained one; that laboratory did practice the modified AOAC method **(16.032)** before the collaborative study.

Verification of Milk-Splitting Accuracy and Milk Quality

When the 9 milks were checked for splitting accuracy by testing for fat in the first, middle, and last bag of milk poured, the range in fat as measured by the Milko-tester **(16.068)** was never greater than 0.02%. The average range across the first, middle, and last bag of milk was 0.01% milkfat. One of the 9 milks had a somatic cell count of 1.1×10^6 cells/mL (material 6). All other milks had a somatic cell count of $\leq 5.5 \times 10^5$ cells/mL; the mean of these 8 milks was 3.3×10^5 cells/mL.

Results of Collaborative Comparison of Total Solids Methods

All data collected for total solids by direct forced air oven drying are reported in Table 1. Blind duplicates have been paired for each sample material. There were no invalid data or AOAC statistical outliers. The mean test results are reported by material. Data collected for total solids by steam table predry are reported in Table 2. Blind duplicates have been paired for each sample material. Again, there were no invalid data. A total of 4 (4 materials, 8 individual test results) AOAC statistical outliers were identified for laboratory A by the Cochran test, which evaluates within-laboratory repeatability, and the Grubbs test, which evaluates between-laboratory averages (extremely high or low values). These statistical outliers appear to be due to a sample mix-up in the testing laboratory.

Results by material and method are summarized in Table 3. The overall difference between methods was 0.0020% total solids, with the direct forced air oven method testing higher. The largest difference between tests was 0.0096% total solids; the smallest difference between tests was 0.0006%. Sherbon et al. (6) found that AOAC method **16.032** gave higher results than a direct forced air oven method (0.035%). This may have been caused by not using blanks in AOAC steam table predry method **16.032** (1). If the blanks had not been used in our study, the steam table predry method would have shown higher results (about 0.036% total solids) as found in the study by Sherbon et al. (6). Therefore, it is very important to include blanks in the testing method to obtain good agreement between the 2 methods.

The rank that each laboratory received in each testing method is indicated in Table 4. Laboratory A was excluded from the ranking because of statistical outliers in the predry method. It is interesting to note that a laboratory that had low results in one method usually had low results for the other method and vice versa. This indicates systematic errors that were common to both methods. If these systematic errors could be identified, it might be possible to improve the between-laboratory reproducibility, however, the source of systematic errors is not known at this time. Some influences on the results of both methods within a single laboratory could have been the oven (temperature and/or heating efficiency) and/or the sample handling (mixing, warming, weighing conditions, etc.). The range of mean test values across laboratories (direct oven 0.115% and modified AOAC 0.127%) could be reduced if the source of the systematic error within laboratories for both methods could be identified and eliminated.

Blank values are shown in Table 5. The blank values for the direct forced air oven method averaged 0.0000 g, with a high of 0.0007 and a low of −0.0003 g. The mean blank value for the steam table predry method was 0.0011 g. The steam table predry blanks were more variable, with a high value of

Table 4. Ranking and mean test result for each laboratory and each method for determination of total solids in raw milk

Lab.	Rank[a]: Direct oven	Rank[a]: Steam predry	Mean % total solids: Direct oven	Mean % total solids: Steam predry
A	—[b]	—	—	—
B	2.1	2.6	12.6897	12.6898
C	2.4	2.8	12.6993	12.6914
D	5.1	3.0	12.7354	12.6924
E	7.3	6.8	12.7587	12.7471
F	5.4	4.3	12.7367	12.7082
G	1.5	2.4	12.6837	12.6897
H	8.7	8.9	12.7983	12.8166
I	5.3	6.1	12.7365	12.7343
J	7.1	8.1	12.7550	12.7707

[a] Rank of 1 = lowest test result; rank of 9 = highest test result.
[b] Not ranked because of outliers.

Table 6. AOAC statistical parameters for each sample material for determination of total solids in raw milk by direct forced air oven drying method

Statistic	Material 1	2	3	4	5	6	7	8	9
Number of labs	10	10	10	10	10	10	10	10	10
Number of accepted values	20	20	20	20	20	20	20	20	20
Mean, %	14.583	12.503	12.405	12.041	12.834	12.325	12.650	12.895	12.327
s_r	0.032	0.025	0.015	0.013	0.025	0.012	0.026	0.017	0.008
RSD_r, %	0.221	0.196	0.124	0.111	0.192	0.095	0.208	0.132	0.062
s_R	0.052	0.038	0.042	0.032	0.034	0.039	0.054	0.047	0.038
RSD_R, %	0.360	0.308	0.341	0.267	0.261	0.314	0.425	0.362	0.309
r value	0.091	0.069	0.043	0.038	0.070	0.033	0.075	0.048	0.022
R value	0.148	0.109	0.120	0.091	0.095	0.110	0.152	0.132	0.108

0.0025 and a low value of 0.0001 g. Thus, the collaborative study substantiated the same trend discovered in the preliminary work: steam table predry blanks were higher and more variable than were direct forced air oven blanks. This indicated clearly that blanks are required for accurate test results in the steam table predry method.

The AOAC statistical parameters for both repeatability and reproducibility for each sample material for the total solids with direct forced air oven drying method are shown in Table 6, and those for the steam table predry method are shown in Table 7. The means of all AOAC statistical parameters for both methods are shown in Table 8. Both methods gave similar overall results and excellent performance. Mean s_r was less than 0.02 for both methods, and mean s_R was less than 0.05 for both methods. These results compare favorably with the earlier study by Green and Park (3), which evaluated the AOAC with steam table predry method. They obtained an s_r of 0.022 and an s_R of 0.068. The r and R values found for both methods in this study were slightly better than those reported in the IDF collaborative study (4). The high somatic cell count milk (material 6) had typical s_r and s_R values.

With only 9 sample materials and a relatively narrow range of total solids content (12.0409–12.8950% for 8 of the 9 materials), it was not reasonable to run linear regressions on total solids content vs any of the AOAC statistical parameters; one unusual value could easily skew the analysis. To determine the influence of raw milk total solids level on any of the AOAC statistical parameters, further research is needed using many more materials distributed more uniformly over the entire range of total solids content of raw milk.

Comparison of Procedures/Limitations

Determining milk total solids with direct forced air oven drying is simpler than with steam table predrying. No additional sample manipulation is needed in the direct forced air oven drying method (such as physically moving the sample to and from a steam table, which incorporates a greater chance of error by the analyst). At 4 h, the total drying time for the direct forced air oven method was 35 min longer than the total drying time for the steam table predry method (25 min predry plus 3 h oven dry). However, in the steam table predry method, one must account for sample manipulation time (moving the samples to the steam bath, placing the samples on and off the steam bath, and moving the samples to the drying oven) and the actual number of samples that a particular steam bath can accommodate at one time (some steam baths accommodate only 8 samples at once). The predry method exposes the sample to the laboratory environment for a longer time and thus increases its exposure to dust and dirt particles in the air, which can cause erroneous test results. Nonuniform conditions from position to position on the steam table may cause more variation in blanks. Total solids by direct forced air oven drying is a more direct method with less chance of error. Repeatability, reproducibility, and mean test value for the direct forced air oven method compare favorably with those for the modification of AOAC method **16.032** when the steam table method includes blank values.

Table 7. AOAC statistical parameters for each sample material for determination of total solids in raw milk by steam table predry method

Statistic	Material 1	2	3	4 All data	4 Outliers excluded	5 All data	5 Outliers excluded	6	7 All data	7 Outliers excluded	8	9 All data	9 Outliers excluded
Number of labs	10	10	10	10	9	10	9	10	10	9	10	10	9
Number of accepted values	20	20	20	20	18	20	18	20	20	18	20	20	18
Mean, %	14.583	12.504	12.396	12.071	12.038	12.806	12.828	12.326	12.626	12.657	12.896	12.342	12.317
s_r	0.021	0.016	0.019	0.147	0.011	0.104	0.018	0.012	0.139	0.013	0.029	0.127	0.018
RSD_r, %	0.142	0.124	0.151	1.218	0.092	0.812	0.142	0.099	1.101	0.102	0.226	1.032	0.150
s_R	0.048	0.049	0.053	0.153	0.042	0.108	0.038	0.051	0.146	0.046	0.047	0.128	0.048
RSD_R, %	0.330	0.388	0.429	1.267	0.353	0.844	0.295	0.417	1.155	0.365	0.367	1.037	0.389
r value	0.058	0.044	0.053	0.416	0.031	0.294	0.051	0.035	0.393	0.036	0.082	0.360	0.052
R value	0.136	0.137	0.151	0.433	0.120	0.360	0.107	0.146	0.413	0.131	0.134	0.362	0.136

Table 8. Mean AOAC statistical parameters for each method for determination of total solids in raw milk

Statistic	Direct forced air oven	Steam table predry
Mean, %	12.7293	12.7273
s_r	0.019	0.017
RSD_r, %	0.149	0.136
s_R	0.042	0.047
RSD_R, %	0.327	0.370
r value	0.054	0.049
R value	0.118	0.133

Recommendations

(*1*) The Associate Referee recommends that the modified description of the method for total solids determination by the steam bath predry method (including blank determinations) be adopted official first action as an improved method to replace method **16.032** for determination of the total solids content of raw milk.

(*2*) The Associate Referee recommends that the method for total solids determination by direct forced air drying be adopted official first action as a new method for determination of the total solids content of raw milk.

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Texas
Upper Midwest

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Combination of Total Solids Determined by Oven Drying and Fat Determined by Mojonnier Extraction for Measurement of Solids-Not-Fat Content of Raw Milk: Collaborative Study

JENNY L. CLARK and DAVID M. BARBANO
Cornell University, Department of Food Science, Ithaca, NY 14853
CHAPMAN E. DUNHAM
Texas Milk Market, PO Box 110939, Carrollton, TX 75011

Collaborating Laboratories: Cornell University; and laboratories operated by or under contract to the following Federal Milk Markets: Chicago Regional; Eastern Ohio/Western Pennsylvania; Greater Kansas City; New England; Southern Illinois-Eastern Missouri; Southwest Plains; Texas; Upper Midwest

Each of 9 laboratories analyzed 9 pairs of blind duplicate raw milk samples for fat, using the Mojonnier ether extraction method (16.E13–16.E17), and for total solids, using a new direct forced air oven method. Solids-not-fat was determined by subtracting percent fat from percent total solids. The solids-not-fat content of the samples tested in the collaborative study ranged from 8.48 to 9.36%. The average repeatability standard deviation (s_r) and the average reproducibility standard deviation (s_R) for the solids-not-fat method were 0.019 and 0.041, respectively. Average repeatability (RSD_r) and reproducibility (RSD_R) relative standard deviations were 0.218 and 0.466%, respectively. The mean repeatability value (r) was 0.055; the mean reproducibility value (R) was 0.117. The difference between milk total solids determined by direct forced air oven drying and milk fat determined by Mojonnier ether extraction has been approved for interim official first action for determination of solids-not-fat content of milk.

Solids-not-fat content of raw milk is determined by subtracting percent fat from percent total solids. In the absence of an AOAC method, laboratories have used a variety of combinations of methods for determining milk fat (e.g., ether extraction, Babcock, etc.) and total solids to determine solids-not-fat content of milk.

Confusion about which method to use for this determination leads to problems in calibrating infrared milk-testing equipment. Infrared test results can only be as accurate as the chemical reference methods used for calibration, so the lack of a uniform reference method can contribute to poor agreement of results among laboratories. An accurate reference method for determining the raw milk solids-not-fat content becomes even more important as producer payment plans change from a system based solely on milk fat content to a multiple component pricing plan based on milk fat and milk solids-not-fat and/or protein content.

Previous research (1) established that, for determining fat content of raw milk, a modified Mojonnier ether extraction method (**16.E13–16.E17**; 1, 2) gave better repeatability and reproducibility than did a Babcock procedure (**16.E10–16.E12**). Thus, the ether extraction method would be more suitable as an infrared reference method. Other recent research (3) has shown that the direct forced air oven drying of milk samples is a highly reliable method for determining milk total solids. That method has received AOAC interim official first action approval (3). The repeatability and reproducibility of solids-not-fat determination by a reference method will be a function of the repeatability and reproducibility of both the fat and total solids testing methods used. It is desirable to combine the best reference methods for fat and total solids to achieve a highly accurate solids-not-fat reference for calibrating infrared milk analyzers. The objectives of the present study were to clearly define the procedure for a chemical reference method to determine raw milk solids-not-fat, and to characterize its repeatability and reproducibility.

Preliminary Work

Nine farms were selected to supply milk for this study, on the basis of a history of good milk quality (i.e., low bacteria counts and low somatic cell counts). Farms were also selected to reflect typical variations in herd size and breed of cow. Seven of the farms had ≥ 98% Holstein herds. One farm had a 100% Jersey herd. One farm had 50% Holstein and 50% Brown Swiss. The mean herd size was 131 cows; the smallest farm consisted of 40 cows, and the largest of 300.

Nine laboratories in different regions of the country participated in the study. Each laboratory conducted both the ether extraction and total solids methods. All laboratories had to be willing to follow the test procedures as outlined and to meet the time constraints necessary for testing unpreserved raw milk. The same technician did not have to conduct both the ether extraction and the total solids procedures in each laboratory.

Other preliminary work by the Associate Referee involved conducting (separately) collaborative studies on the ether extraction method (**16.E13–16.E17**; 1, 2) and total solids method (3). Previous collaborative studies characterized the repeatability and reproducibility of the fat and total solids methods individually. The results of those previous studies provide the reference points for the solids-not-fat method.

Collaborative Study

Design of the Study

For the collaborative study, each of 9 laboratories received 2 different sets of 18 raw milk samples (9 pairs of blind duplicates). Each pair of blind duplicates represented one farm, and included blind duplicates within methods, between methods (fat and total solids), and among laboratories. The testing order was random between methods and laboratories. The central computing laboratory calculated solids-not-fat results after matching blind duplicates for ether extraction and total solids.

A 3-digit sample coding system was used. Each laboratory simultaneously received 1 set of 18 milk samples for fat determination and a second set of the same 18 milk samples (coded differently) for total solids determination. Because of the time required to conduct fat or total solids tests and to avoid excessive or double warming of raw milk samples prior to testing, 2 independent sets of samples were used. One data form accompanied each set of 18 milk samples; all the sample

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The recommendation has been approved interim official first action by the General Referee, the Committee on Foods I, and the Chairman of the Official Methods Board. The method will be submitted for adoption official first action at the 103rd AOAC Annual International Meeting, September 25–28, 1989, at St. Louis, MO. Association actions will be published in "Changes in Official Methods of Analysis" (1990) *J. Assoc. Off. Anal. Chem.* **73**, January/February issue.

Table 1. Percent total solids content of raw milk determined by direct forced air oven method (3)

	Laboratory									Material
Material	A	B	C	D	E	F	G	H	I	mean
1	15.0110	15.0135	15.0241	14.9875	15.0041	15.0074	15.0003	15.0916	15.0257	15.0077
	14.9890	15.0252	15.0280	14.9907	14.9505	14.9691	14.9641	15.0585	14.9944	
2	12.2083	12.2136	12.2692	12.1771	12.1752	12.2321	12.2346	12.2310	12.2621	12.2261
	12.2033	12.2681	12.2339	12.1772	12.1960	12.2402	12.2310	12.2618	12.2501	
3	12.7525	12.8313[a]	12.8189	12.7632	12.7326	12.8010	12.7857	12.7813[b]	12.8274	12.7796
	12.7490	—[a]	12.8330	12.7388	12.7488	12.7856	12.7773	14.3503[b]	12.8005	
4	12.3126	12.3777	12.4170	12.3250	12.3551	12.3713	12.3780	12.4082	12.3823	12.3762
	12.3567	12.4361	12.4079	12.3349	12.3420	12.3853	12.3684	12.4123	12.4010	
5	12.7889	12.8712	12.8553	12.7867	12.7813	12.7995	12.8056	12.8633	12.8451	12.8176
	12.7780	12.8246	12.8696	12.8056	12.7667	12.8087	12.8188	12.8479	12.7997	
6	12.2369	12.3459	12.3061	12.2362	12.2630	12.2554	12.2820	12.2720	12.2635	12.2736
	12.2464	12.3203	12.3105	12.2457	12.2611	12.3089	12.2698	12.2621	12.2386	
7	12.2808	12.3374	12.3551	12.2472	12.2950	12.3390	12.3045	12.3140[c]	12.3292	12.3145
	12.2891	12.3222	12.3475	12.2622	12.3254	12.3259	12.3279	12.4049[c]	12.3430	
8	12.9849	13.0649	13.0445	12.9908	12.9606	12.9928	13.0172	13.0378	13.0624	13.0167
	13.0117	13.0331	13.0614	12.9844	12.9660	12.9992	13.0213	13.0055	13.0613	
9	12.3908	12.4356	12.4452	12.3450	12.3797	12.3801	12.4024	12.4203	12.3818	12.3927
	12.3524	12.4175	12.4036	12.3317	12.3393	12.3845	12.4072	12.4253	12.4268	

[a] Invalid data (pan dropped); not included in material mean.
[b] Cochran and Grubbs statistical outlier; not included in material mean.
[c] Cochran statistical outlier; not included in material mean.

numbers (3-digit code) were printed on it in the order that the analyst was to test the samples. The forms also identified the method (fat or total solids) by which the analyst would test each set of 18 samples. A form for the analyst to write comments about the testing of each individual sample was also supplied.

In addition, a questionnaire was developed for each testing method and was included with every set of samples. The questionnaire was to be completed by the person conducting the test. Information such as sample arrival time, start and finish time for each method, and actual testing conditions during analysis was requested on the questionnaires to help ensure that the analysts followed all the details of the procedures. Completed questionnaires were returned to the central data processing laboratory with the data sheets. A computer program was prepared to collect data and to translate the sample codes to match blind duplicate test results for both methods (ether extraction and total solids), calculate the solids-not-fat test results, and conduct outlier tests followed by calculation of AOAC statistical parameters (4, 5).

The week prior to the collaborative study, 162 six oz Whirl-Pak bags were coded with sample identification numbers and id numbers were checked. Milk (5 L) was collected from each of 9 farms on a Monday morning (day 1) and transported on ice to the central laboratory. Milk samples were cold split in the central laboratory: the milk from each farm was mixed, poured in one large plastic container, and agitated continuously with a motor-driven stirrer while the milk was drawn from a spout (at the bottom of the container) directly into sample bags. Forty-six or more 6 oz Whirl-Pak bags of milk were prepared for each farm (80 mL per bag). Sample bags were refrigerated immediately after splitting. Sample splitting uniformity was verified immediately after each of the nine 5 L batches of milk was split by checking the fat test of the first, middle, and last sample bag for each batch of milk (a Milko-tester MK III (**16.068**) (6) was used).

Table 2. Statistical parameters for repeatability and reproducibility of total solids determination in raw milk by material

	Material										
	1	2	3		4	5	6	7		8	9
Statistic			All[a] data	Outlier excluded				All data	Outlier excluded		
Number of labs	9	9	8	7	9	9	9	9	8	9	9
Number of accepted values	18	18	16	14	18	18	18	18	16	18	18
Mean, %	15.008	12.226	12.878	12.780	12.376	12.816	12.274	12.319	12.314	13.017	12.393
s_r	0.022	0.017	0.392	0.012	0.019	0.016	0.016	0.024	0.012	0.013	0.020
RSD_r, %	0.143	0.141	3.047	0.096	0.152	0.136	0.131	0.198	0.100	0.102	0.164
s_R	0.034	0.032	0.394	0.034	0.035	0.034	0.033	0.037	0.032	0.035	0.035
RSD_R, %	0.227	0.259	3.059	0.270	0.282	0.267	0.268	0.301	0.264	0.266	0.280
r value	0.061	0.049	1.110	0.035	0.053	0.050	0.045	0.069	0.035	0.038	0.057
R value	0.096	0.090	1.114	0.098	0.099	0.097	0.093	0.105	0.092	0.098	0.098

[a] One invalid data point removed.

Table 3. Comparison of AOAC statistical parameters for repeatability and reproducibility of total solids, fat, and solids-not-fat determinations in raw milk

AOAC statistic	Total solids	Fat	Solids-not-fat
Mean, %	12.801	3.934	8.865
s_r	0.017	0.010	0.019
RSD_r, %	0.130	0.264	0.218
s_R	0.034	0.015	0.041
RSD_R, %	0.265	0.380	0.466
r value	0.047	0.030	0.055
R value	0.096	0.042	0.117

On Monday afternoon, when the milk from all 9 farms had been split, the samples were appropriately grouped, packed in ice, placed in insulated shipping containers, and sent by overnight air delivery to participating laboratories. On Tuesday (day 2), samples arrived at participating laboratories and testing began as soon as possible. Sample temperature on arrival at all laboratories was less than 4°C. All testing was completed by Friday (day 5). Test results and completed questionnaires arrived at the data analysis laboratory by the following Tuesday (day 9). All data were summarized and returned to individual testing laboratories by the next Wednesday (day 17). Collaborators verified that the data compiled for their laboratory were correct.

Milk Quality Verification

A set of milk samples was tested for somatic cell count with a Foss-o-Matic electronic somatic cell counter (7), which was calibrated by direct microscopic somatic cell count (8). This set of samples had been exposed to all the same handling and shipping as the samples that were tested by fat and total solids methods.

Methods

Collaborators were instructed to determine percent total solids of raw milk according to the direct forced air oven method as described in a previous report (3), and percent fat according to the Mojonnier ether extraction method (**16.E13–16.E17**; 2).

Solids-Not-Fat in Milk

By Difference Between Total Solids and Fat Contents

Interim First Action

Method Performance:

$s_r = 0.019$; $s_R = 0.041$; $RSD_r = 0.218\%$; $RSD_R = 0.466\%$

A. Principle

Total solids and fat contents of milk are determined. Percent fat is subtracted from percent total solids to obtain percent solids-not-fat.

B. Determination

(a) *Total solids.*—Determine total solids as in direct forced air oven method (JAOAC **72,** September/October issue (1989)).

(b) *Fat.*—Determine fat as in **16.E13–16.E17**.

C. Calculation

Solids-not-fat, % = % total solids − % fat

Maximum recommended difference between duplicates = 0.06% solids-not-fat.

Ref.: JAOAC **72,** September/October issue (1989).

Results and Discussion

Verification of Milk Splitting Accuracy and Milk Quality

When the 9 batches of milk were tested for splitting accuracy by checking the fat test of the first, middle, and last bag of milk poured, the range in fat as measured by the Milkotester (**16.068**) was never greater than 0.02%. The average range across the first, middle, and last bag of milk was 0.01% milk fat. One of the 9 milks had a somatic cell count of 1.19×10^6 cells/mL (material 6). All other milks had a somatic cell count of 8.42×10^5 cells/mL or less; the mean of these 8 milks was 4.99×10^5 cells/mL. In spite of the high somatic cell count, the test results for material 6 were comparable to the lower somatic cell count samples.

Table 4. Percent fat content of raw milk determined by method 16.E13–16.E17

	Laboratory									
Material	A	B	C	D	E	F	G	H	I	Material mean
1	5.6421	5.6155	5.6524	5.6721	5.6622	5.6360	5.6352	5.6564	5.6477	5.6443
	5.6592	5.6353	5.6247	5.6236	5.6625	5.6509	5.6104	5.6611	5.6506	
2	3.7597	3.7218	3.9321[a]	3.7305	3.7669	3.7294	3.7385	3.7505	3.7543	3.7464
	3.7625	3.7345	3.7348[a]	3.7674	3.7648	3.7357	3.7365	3.7449	3.7448	
3	4.0251	4.0081	4.0218	4.0583	4.0318	4.0110	3.9853	3.9891	4.0000	4.0140
	4.0234	4.0084	4.0026	4.0151	4.0294	4.0115	3.9962	4.0231	4.0109	
4	3.5119	3.5048	3.5120[a]	3.5441	3.5223	3.5067	3.4935	3.5118	3.5064	3.5094
	3.5197	3.5045	3.8690[a]	3.5135	3.5022	3.5081	3.4801	3.5169	3.5044	
5	3.8924	3.8668	3.8715	3.8855	3.8867	3.8708	3.8369	3.8799	3.8731	3.8743
	3.8952	3.8687	3.8651	3.8783	3.8798	3.8715	3.8605	3.8925	3.8623	
6	3.4768	3.4727	3.4664	3.4954	3.4593	3.4717	3.4576	3.4699	3.4706	3.4723
	3.4846	3.4738	3.4692	3.4807	3.4838	3.4812	3.4509	3.4689	3.4676	
7	3.6210	3.5714	3.5778	3.6095	3.6227	3.5985	3.5989	3.5899[b]	3.6125	3.6016
	3.6259	3.5850	3.5931	3.5987	3.6121	3.6048	3.5899	—[b]	3.6036	
8	3.9277	3.8999	3.9323	3.9306	3.9203	3.9210	3.9185	3.9058	3.9192	3.9249
	3.9388	3.9179	3.9292	3.9461	3.9406	3.9400	3.8994	3.9248	3.9358	
9	3.6301	3.6218	3.6220	3.6303	3.6307	3.6068	3.5912	3.6104	3.6130	3.6195
	3.6284	3.6082	3.6060	3.6462	3.6316	3.6337	3.5981	3.6221	3.6203	

[a] Cochran and Grubbs statistical outlier; not included in material mean.

[b] Invalid data (broken flask); not included in material mean.

Table 5. Statistical parameters for repeatability and reproducibility of fat determination in raw milk by material

	Material										
	1	2		3	4		5	6	7[a]	8	9
Statistic		Orig. data	Outlier excluded		Orig. data	Outlier excluded					
Number of labs	9	9	8	9	9	8	9	9	8	9	9
Number of accepted values	18	18	16	18	18	16	18	18	16	18	18
Mean, %	5.644	3.756	3.746	4.014	3.530	3.509	3.874	3.472	3.602	3.925	3.620
s_r	0.016	0.047	0.010	0.014	0.085	0.010	0.007	0.008	0.007	0.012	0.010
RSD_r, %	0.286	1.264	0.275	0.354	2.399	0.286	0.191	0.218	0.205	0.298	0.265
s_R	0.018	0.046	0.015	0.017	0.086	0.014	0.014	0.011	0.016	0.014	0.014
RSD_R, %	0.314	1.226	0.402	0.434	2.429	0.396	0.373	0.311	0.448	0.350	0.395
r value	0.046	0.134	0.029	0.040	0.240	0.028	0.021	0.021	0.02	0.033	0.027
R value	0.050	0.130	0.043	0.049	0.243	0.039	0.041	0.031	0.046	0.039	0.040

[a] One invalid data point removed.

Total Solids Determination

All data collected for the total solids procedure are reported in Table 1. Blind duplicate results have been paired for all laboratories. One set of data from laboratory B (material 3) was invalid because the analyst dropped the pan. There were 2 statistical outliers in laboratory H (materials 3 and 7); both were identified as outliers by the Cochran test. The data for material 3 was also identified as an outlier by the Grubbs test. The mean test results for all laboratories are reported by material. AOAC statistical parameters for the total solids determination are summarized by material in Table 2.

Means of the AOAC statistical parameters for all materials are reported in Table 3 for comparison to past studies and to other methods. The means of the total solids statistical parameters are slightly better than those reported in a previous study (3). (In the previous study of total solids determination, s_r, RSD_r, s_R, RSD_R, r value, and R value were 0.019, 0.149%, 0.042, 0.327%, 0.054, and 0.118, respectively.)

Fat Determination

All data collected for the ether extraction procedure are reported in Table 4. Blind duplicate results have been paired for all laboratories. One set of data in laboratory H (material 7) was invalid because of a broken flask. There were 2 statistical outliers in laboratory C (materials 2 and 4); both results were identified as outliers by both the Cochran and Grubbs outlier tests. The mean test results are reported by material in Table 4. AOAC statistical parameters are summarized by material in Table 5. Means of the AOAC statistical parameters for all materials are reported in Table 3. The means of the ether extraction statistical parameters are slightly better than those reported in a previous study (1). (In the previous

Table 6. Percent solids-not-fat content of raw milk determined by difference (% total solids − % fat)

	Laboratory									Material
Material	A	B	C	D	E	F	G	H	I	mean
1	9.3690	9.4011	9.3716	9.3153	9.3419	9.3714	9.3651	9.4352	9.3780	9.3634
	9.3298	9.3909	9.4033	9.3671	9.2880	9.3182	9.3537	9.3974	9.3438	
2	8.4486	8.4968	—[a]	8.4466	8.4083	8.5027	8.4961	8.4805	8.5078	8.4765
	8.4408	8.5336	—[a]	8.4098	8.4312	8.5045	8.4945	8.5169	8.5053	
3	8.7274	—[b]	8.7971	8.7049	8.7008	8.7900	8.8004	—[c]	8.8274	8.7637
	8.7256	—[b]	8.8304	8.7237	8.7194	8.7741	8.7811	—[c]	8.7896	
4	8.8006	8.8729	—[a]	8.7810	8.8328	8.8646	8.8845	8.8965	8.8759	8.8623
	8.8370	8.9316	—[a]	8.8214	8.8398	8.8772	8.8883	8.8954	8.8966	
5	8.8965	9.0044	8.9838	8.9012	8.8946	8.9287	8.9688	8.9835	8.9720	8.9433
	8.8828	8.9559	9.0046	8.9273	8.8869	8.9372	8.9583	8.9553	8.9374	
6	8.7601	8.8741	8.8397	8.7408	8.8037	8.7837	8.8244	8.8021	8.7929	8.8013
	8.7618	8.8464	8.8413	8.7651	8.7773	8.8277	8.8189	8.7932	8.7710	
7	8.6598	8.7660	8.7774	8.6377	8.6723	8.7404	8.7056	—[d]	8.7167	8.7129
	8.6632	8.7372	8.7544	8.6635	8.7133	8.7212	8.7380	—[d]	8.7394	
8	9.0571	9.1650	9.1122	9.0602	9.0403	9.0719	9.0988	9.1321	9.1432	9.0918
	9.0729	9.1153	9.1322	9.0383	9.0254	9.0593	9.1219	9.0807	9.1255	
9	8.7607	8.8138	8.8232	8.7147	8.7490	8.7732	8.8112	8.8098	8.7689	8.7732
	8.7240	8.8093	8.7977	8.6855	8.7077	8.7508	8.8091	8.8031	8.8066	

[a] Cochran and Grubbs statistical outlier from fat determination.

[b] Invalid data from total solids determination.

[c] Cochran statistical outlier from total solids determination.

[d] Cochran and Grubbs statistical outlier from total solids and invalid data from fat determinations.

Table 7. Statistical parameters for repeatability and reproducibility of solids-not-fat determination in raw milk by material

Statistic	Material 1	2[a]	3[b]	4[a]	5	6	7[c]	8	9
Number of labs	9	8	7	8	9	9	8	9	9
Number of accepted values	18	16	14	16	18	18	16	18	18
Mean, %	9.363	8.476	8.764	8.862	8.943	8.801	8.713	9.092	8.773
s_r	0.028	0.017	0.017	0.021	0.018	0.016	0.019	0.020	0.019
RSD_r, %	0.296	0.201	0.190	0.237	0.202	0.181	0.216	0.224	0.218
s_R	0.037	0.041	0.047	0.041	0.040	0.037	0.043	0.042	0.044
RSD_R, %	0.394	0.479	0.533	0.463	0.452	0.420	0.496	0.460	0.501
r value	0.078	0.048	0.047	0.059	0.051	0.045	0.053	0.058	0.054
R value	0.104	0.115	0.132	0.116	0.114	0.105	0.122	0.118	0.124

[a] Data discarded—statistical outlier in fat analysis.
[b] Data discarded—invalid data and statistical outlier in total solids analysis.
[c] Data discarded—statistical outlier in total solids analysis and invalid data in fat analysis.

study of fat determination, s_r, RSD_r, s_R, RSD_R, r value, and R value were 0.015, 0.396%, 0.020, 0.512%, 0.044, and 0.056, respectively.)

Solids-Not-Fat Determination

All data collected for the solids-not-fat method are reported in Table 6. Five data points were invalid. No invalid data or statistical outliers were discarded for the solids-not-fat method other than those previously identified for total solids (Table 1) and fat (Table 4). AOAC statistical parameters are summarized by material in Table 7. Means of the AOAC statistical parameters for all materials are reported in Table 3. As expected, repeatability and reproducibility of the solids-not-fat method were not as good as those for either the total solids or the ether extraction methods. Since the solids-not-fat result is derived from independent total solids and fat determinations, one would not expect to obtain better repeatability and reproducibility than that of its components (total solids and fat). Regressions of AOAC statistical parameters vs percent solids-not-fat were not conducted because such a small population of samples was involved. One unusual value would easily have skewed the curve.

Ranking of Laboratories Within Each Method

Table 8 indicates the high/low ranking of laboratories in each testing method, calculated as in Youden (4). Laboratories with outlier or invalid data were not included in the ranking of laboratories by total mean test value. It is noted that a laboratory with a low total solids mean test value tended to have a high fat test value and vice versa. The samples for the total solids and fat determinations were taken from separate sample containers for each sample material, so the ranking behavior is not caused by a sequential sampling error from the same container. A systematic factor(s) common to both methods may have had an opposite effect (between the methods) on the fat and total solids test results. If this factor could be identified and eliminated, the high/low range in tests between laboratories could be decreased (resulting in better between-laboratory reproducibility). It is not known at this time what influenced the test results. Some possible influences could have been differences in ovens (temperature and/or heating efficiency) and/or sample handling (i.e., mixing, warming, weighing conditions, etc.). Further research is needed to identify the source of this systematic error. As expected, the laboratory ranking of solids-not-fat results was influenced the most by the total solids ranking.

Recommendation

The Associate Referee recommends that a combination of the Mojonnier ether extraction for fat determination (**16.E13–16.E17**) and the forced air oven drying method for total solids determination (3) be adopted official first action for determination of the solids-not-fat content of milk.

Acknowledgments

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Chicago Regional
Eastern Ohio/Western Pennsylvania
Greater Kansas City
New England
Southern Illinois-Eastern Missouri
Southwest Plains

Table 8. Laboratory ranking and mean test values for laboratories with no invalid or outlier data for solids-not-fat determination in raw milk

Lab.	Rank[a] Total solids	Rank[a] Fat	Rank[a] Solids-not-fat	Mean test, % Total solids	Mean test, % Fat	Mean test, % Solids-not-fat
A	2.8	5.0	2.6	12.7746	3.9458	8.8288
B[b]	—	—	—	—	—	—
C[b]	—	—	—	—	—	—
D	1.9	4.8	1.7	12.7628	3.9459	8.8169
E	2.0	4.7	2.0	12.7690	3.9450	8.8240
F	4.4	2.7	4.3	12.7992	3.9327	8.8665
G	4.3	1.1	5.2	12.7998	3.9154	8.8844
H[b]	—	—	—	—	—	—
I	5.6	2.7	5.2	12.8164	3.9332	8.8832

[a] Rank of 1 = lowest testing laboratory.
Rank of 6 = highest testing laboratory.
[b] No ranking or mean test results because of invalid and/or outlier data points.

Texas
Upper Midwest

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Alternative Methods for Infrared Analysis of Fat in Milk: Interlaboratory Study

DELMAR A. BIGGS
Biggs Consulting, 3050 Keighly Rd, Nanaimo, British Columbia V9T 2X8, Canada
DAVID McKENNA
Foss Food Technology Corp., 25710 Velan Dr, Valencia, CA 91355

Collaborators: C. Christenson; J. Michelson; L. Szijarto

A study in 3 laboratories has demonstrated that accuracy of infrared analysis of fat in milk can be improved relative to results by AOAC method 16.083-16.092 by measuring IR absorption by CH_2 and CH_3 groups in fat molecules instead of or in addition to measuring absorption by carbonyl groups. Theoretical implications for alternative choices of measurements for fat analysis are discussed, and data are presented to indicate the agreement between theory and practice. Mathematical formulas developed from the Beer-Lambert law are presented to describe mechanisms for dual wavelength, double beam, and single beam optical arrangements. A mathematical description of circuitry for these arrangements is included. Methods for multiple linear regression estimates of calibration parameters are discussed. Comparison data indicate optimum situations regarding frequency of calibration and random selection of calibration samples. With optimum control of calibration parameters, mean bias for randomly chosen check samples was reduced to <0.01%. Standard deviation of difference for fat estimates was reduced to range from 0.026 to 0.034%. Quality control procedures and calibration techniques for individual instruments are described. The use of the Fat B filter and a combination of the Fat A and Fat B filters has been adopted official first action as a revision in method 16.083-16.092.

Since 1972, when an infrared method for milk analysis was first adopted by AOAC, efforts to improve the speed and accuracy of this method have led to many changes in instrumentation and procedure. By 1978, it became obvious, considering the relatively high cost of these instruments, that it would be impractical to require an AOAC collaborative study for each new model of each instrument. Accordingly, in 1979 a set of performance specifications was prepared for assessment and approval of future instrument models (1). These specifications set maximum limits for the mean difference and standard deviation of difference for both comparisons between duplicate instrument estimates and comparisons between instrument estimates and results of standard reference methods.

The chemical structure of the fat molecule is illustrated in Figure 1. The fatty acid chains, which account for approximately 73% (by weight) of fat in the molecule, are connected to the glycerol and carbonyl portion of the molecule by ester linkages. The glycerol and carbonyl portion accounts for approximately 27% (by weight) of milk fat in the molecule. The A-type measurement, AOAC method **16.083-16.092** (2), counts the ester linkages by measuring the absorption of infrared radiation at 5.723 μm. The A-type measurement can thus be described as a molecule counter, which is insensitive to chemical changes in fat composition that occur in the fatty acid chains.

The accuracy by which the fat concentration in a milk sample can be measured is a function of (*1*) variations in chain length that cause changes in the molecular weight of the fat in the sample, and (*2*) variations in levels of unsaturation of the fat in the sample. The errors associated with these variations were the subject of major research programs by instrument manufacturers in the mid-1970s. In 1981, a U.S. patent, which described the B-type measurement, was published by Nexo et al. (3). B-type measurement uses the CH_2 and CH_3 absorption centers in the fatty acid chains, which, as already stated, represent approximately 73% of the fat by weight in the molecule and are sensitive to the variations in chemical composition described above.

With respect to the variations in molecular weight, the B-

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The recommendation was approved interim official first action by the General Referee, the Committee on Foods I, and the Chairman of the Official Methods Board, and was adopted official first action at the 101st AOAC Annual International Meeting, September 14-17, 1987, at San Francisco, CA (use of A, B, or A + B filters) and 102nd AOAC Annual International Meeting, August 29-September 1, 1988, at Palm Beach, FL (calibration procedures). Association actions were published in "Changes in Official Methods of Analysis" (1988) *J. Assoc. Off. Anal. Chem.* **71,** January/February issues and (1989) *J. Assoc. Off. Anal. Chem.* **72,** January/February issue.

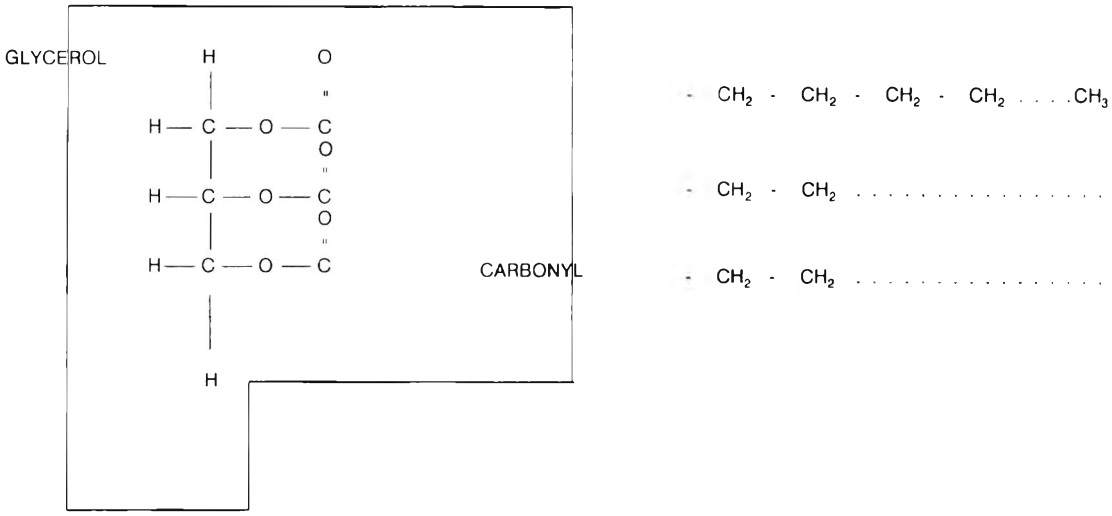

Figure 1. Chemical structure of fat molecule.

type measurement, which measures the absorption of IR radiation at 3.48 μm, actually measures the length of the fatty acid chain. Because of this, and also because the fatty acid chains represent the majority of the fat in the molecule, we will demonstrate theoretically that the B-type method of analysis is substantially more accurate than the A-type method in overcoming chemical variations in molecular weight. The results of the present study confirm this. Because neither A- nor B-type absorptions truly represent the whole molecule, the (A + B) technique was developed and is the subject of a U.S. patent published by Biggs et al. (4) in 1984. We will show that, theoretically, with respect to molecular weight variations, the accuracy of the combined (A + B) measuring technique is almost totally independent because the whole molecule is represented in the measurement.

Unsaturation, which is illustrated in Figure 2, is essentially the presence of a double bond in the fatty acid chain and the loss of 2 hydrogen atoms. We will also show theoretically that the variation in molecular weight due to the loss of 2 hydrogens will be essentially insignificant with respect to the A measurement, and that this method of analysis is nearly independent of chemical variations of this type. On the other hand, the loss of the CH_2 absorption centers cause the B-type method to be dependent on the fat composition. In practice, the work performed by Sjaunja demonstrated that variations in unsaturations are, in many cases, naturally compensated by opposing effects of variations in molecular weight (L. O. Sjaunja, University Agricultural Sciences, Upsala, Sweden, 1987, personal communication). For this reason, the B-type measurement maintains its supremacy over the A-type measurement.

For the reasons already outlined, it is only to be expected that the (A + B) method will prove to be significantly more accurate than the Fat A method and slightly more accurate than the Fat B method.

Both of these modified methods, Fat B and (A + B), are being used in the milk industry and users appear to be satisfied that accuracy estimates are well within the aforementioned performance specifications. However, since both measurements use a wavelength not previously covered by AOAC method **16.083–16.092,** they represent a change in methodology. It is imperative that the AOAC method be updated to include them.

We decided to include a comprehensive theoretical description of the mechanisms involved in the measurement and also include equations that were derived from the basic law to demonstrate the techniques involved in overcoming the interference effects encountered with aqueous systems and the respective intercomponent interferences.

Theoretical Analytical Model for Fat A vs Fat B vs Fat (A + B)

Although absorption intensities of CH_2 stretching vibrations (Fat B) are known to be proportional to chain length in long chain paraffins, the absorption by individual CH_2 groups may be decreased if they are adjacent to a carbon-to-carbon double bond or are adjacent to a carbonyl group, or if the compound contains oxygen (5). To investigate these effects in triglycerides, a homologous series of triglycerides from tributyrin to tristearin plus the 3 unsaturated triglycerides, triolein, trilinolein, and trilinolenin, were used to prepare solutions in chloroform followed by measurement of their relative absorptivities at 3.48 μm with a Beckman Model 4230 infrared spectrometer. Relative absorptivities increased progressively with increasing chain length, but decreased substantially with each successive increase in unsaturation, indicating that the presence of one or more double bonds in the fatty acid chain significantly reduces absorption by CH_2 groups. Increases in molecular weight of triglycerides, because they involve increases in chain length of the substituent fatty acids, increase absorptivity.

On the other hand, increases in molecular weight of triglycerides cause decreases in absorptivity at the Fat A waveband (5.723 μm) because of the relatively constant absorption of the 3 carbonyl groups in the molecule. The fact that changes in molecular weight have the opposite effect at the Fat A and Fat B wavelengths leads to the conclusion that by judicious choice of portions of both Fat A and Fat B signals one could essentially eliminate most of the variability caused by changing molecular weight. If the Fat A absorption of carbonyl groups is designated as representing the glycerol portion of the molecule plus the ester linkages and carbonyl groups, leaving the rest of the molecule to be represented by the CH_2 absorption, one can calculate for each kind of fat the approximate portion of each signal to choose, using the average molecular weight of the fat. In effect, the Fat A signal

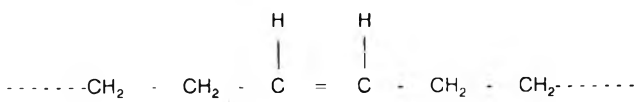

Figure 2. Fatty acid chain.

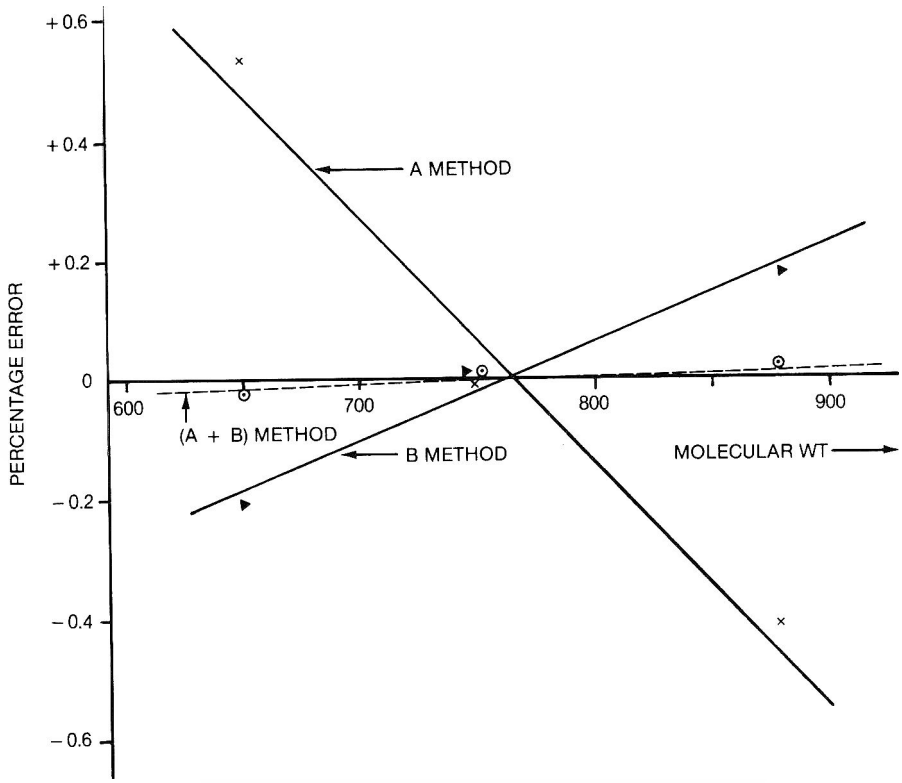

Figure 3. Error due to molecular weight variations for a 3.5% fat sample. Calculations for the (A + B) method used 27% A and 73% B.

would be measuring only the invariable portion of the triglyceride molecules. There still would be interference with the Fat B signal as a measure of the rest of the molecule because changes in unsaturation tend to be seasonal, so that within season, there should be less variation from this source providing the calibration is adjusted frequently to the current requirement for the milk population being tested. An advantage of the combined Fat A and Fat B calibration is that the interference coefficients for protein and lactose are smaller than for the Fat B equation because both Fat A coefficients are positive and both Fat B coefficients are negative.

A simple model can be used to clearly illustrate how these different mechanisms affect the quantitative analysis of fat by the infrared method. The effect of changes in molecular weight is magnified by selecting oils that cover a much wider range than would be found in the normal variations in butter-

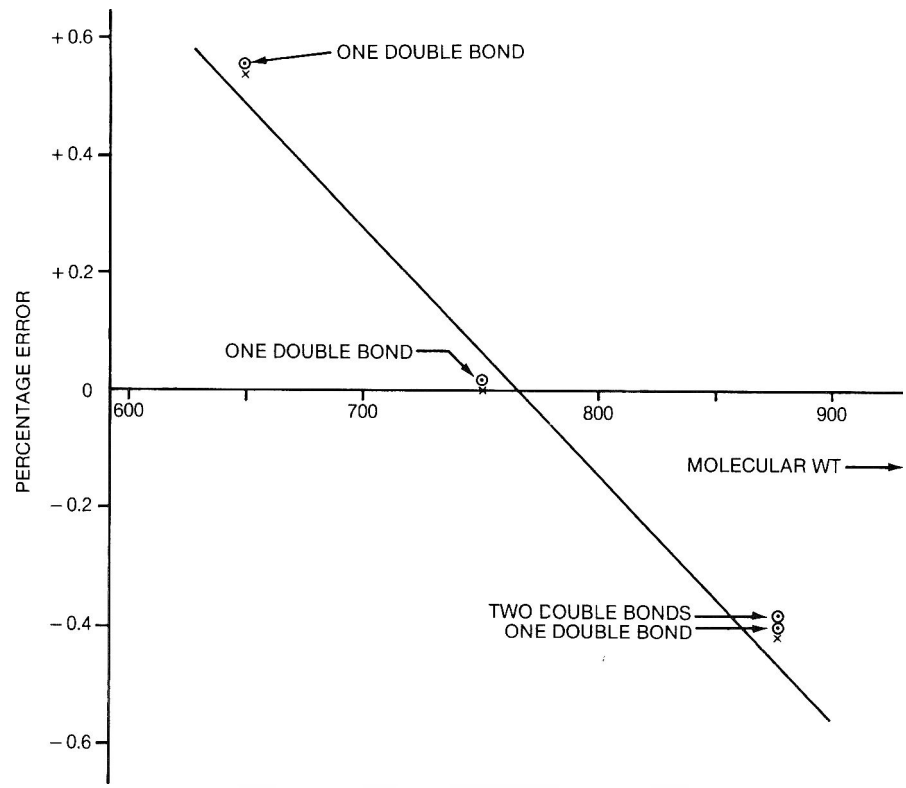

Figure 4. Effect of unsaturation on a 3.5% fat sample using Fat A method.

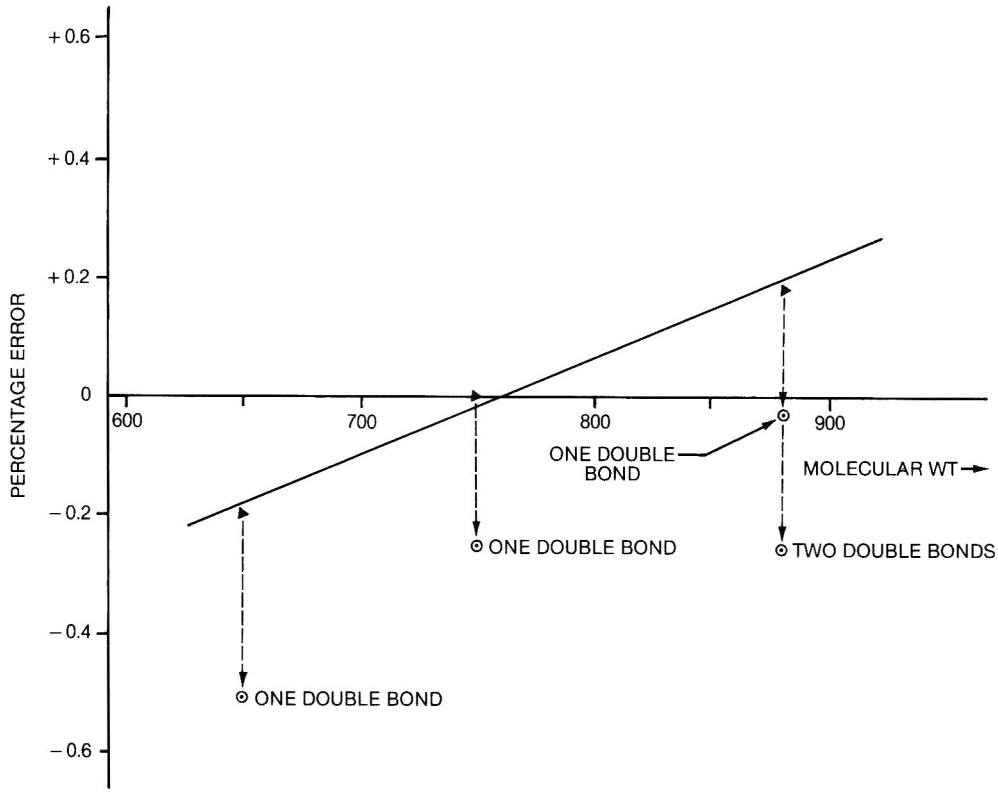

Figure 5. Effect of unsaturation on a 3.5% fat sample using Fat B method.

fat. The oils used in the model are coconut oil (MW 650) and soybean oil (MW 880) compared to butterfat with an average molecular weight of 750.

The effect of these molecular weight variations on the A-type measurement for samples containing a 3.5% concentration of these different oils was calculated for an instrument that had been calibrated for butterfat. We assumed in the first instance that the fats in these samples were completely saturated so the errors involved can be attributed to the change in molecular weight. A similar calculation was performed for the B and (A + B) measurements. The percentage error as a function of molecular weight (Figure 3) shows that both the A and B methods are sensitive to changes in molecular weight but that the changes are of opposite sign. For the

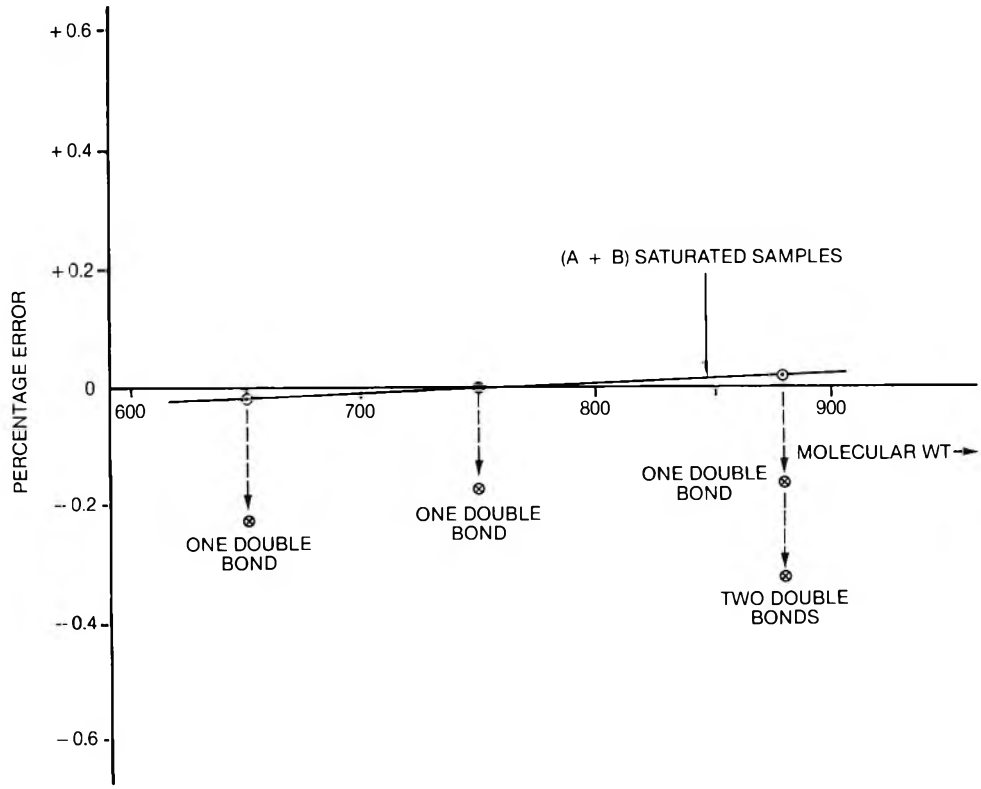

Figure 6. Effect of unsaturation on a 3.5% fat sample using Fat (A + B) method. Calculation used 27% A and 73% B.

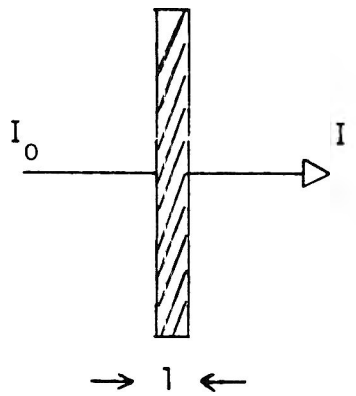

Figure 7. Illustration of Beer-Lambert law: $I = I_0 e^{-kcl}$, where I_0 is incident radiation intensity; I is transmitted radiation intensity; l is sample path length; c is concentration; and k is extinction coefficient, which is constant at a particular wavelength.

same change, the magnitude of the B measurement error is less than that for the A by a factor of about three. The (A + B) method is almost insensitive to these changes.

The effect of unsaturation was next introduced using the same oils with the same calibration. The changes in error by the introduction of a single double bond for the 3 samples and 2 double bonds for the soybean oil were calculated. The results of the calculations are shown in Figure 4 for the A-type measurement, Figure 5 for the B-type measurement, and Figure 6 for the combination (A + B). It can be seen that the B-type measurement is the most sensitive to changes in levels of unsaturation, followed by (A + B), which is improved by the presence of the A measurement, which is almost insensitive to these variations. Regardless of the theoretical implications regarding the relative accuracies of the A, B, and (A + B) calibration methods, the final evidence in this regard must come from comparisons of actual results using these methods on the same milks. These data have been gathered and are presented in the results section of this paper.

Mathematical Model of Absorption Mechanisms

So far we have considered only the absorption of infrared radiation by the fat molecule in a single component system. When other components are present, namely, protein and lactose, for a fixed concentration of any one of these components, the magnitude of the absorption at its characteristic wavelength is modified by the level of concentration of the other 2 components (6). This interaction is commonly referred to as intercomponent interference; the different mechanisms involved can be described with the help of a mathematical model.

Published optical transmission spectra (6) show that strong absorption due to water occurs at all the wavelengths of interest, and that the absorption bands representing the other components of milk are superimposed on this water background. Over the past 20 years, different manufacturers of infrared instruments have developed a number of optical systems of different design. Those still in common use can be described as follows:

(*1*) Single beam/single wavelength, where the absorption of milk at each component wavelength is referenced to the absorption of water at the same wavelength (Multispec Dairylab 1, Multispec Ltd, Wheldrake, York, UK).

(*2*) Single beam/dual wavelength, where the absorption of milk at each component wavelength is referenced to the absorption of milk at a suitable wavelength adjacent to the absorption band (7).

(*3*) Double beam/dual wavelength, where the absorption of the milk at each component wavelength is referenced to the absorption of milk at a suitable wavelength adjacent to the absorption band (4).

These different optical configurations require a separate mathematical derivation to describe the relationship between the major absorptions, which are used for measurement, and the minor absorptions, which are the source of interference. The basic equations for single and double beam/single wavelength and single and double beam/dual wavelength are described below.

The basic law is illustrated in Figure 7. It can be shown, using the mathematical treatment described by McKenna (8), for single and double beam/single wavelength operation, the following equation describes the absorption mechanisms at the Fat A or Fat B wavelengths:

$$\ln(I_r/I_s) = k_f F l - k_{wf}\phi l + (k_p P l - k_{wf}\gamma l) + (k_l L l - K_{wf}\delta l) \ldots \quad (1)$$

where l is the cell path length.

F, P, and L are the concentrations of fat, protein, and lactose, respectively.

ϕ, γ, and δ are functions of the fat, protein, and lactose concentrations, respectively.

k_f, k_p, k_l, and k_{wf} are the extinction coefficients at 5.723 or 3.48 μm for fat, protein, lactose, and water, respectively.

$k_f F l$ is the absorption due to fat.

$k_{wf}\phi l$ is the absorption due to water displacement by fat.

$k_p P l - k_{wf}\gamma l$ = net interference due to protein.

$k_l L l - k_{wf}\delta l$ = net interference due to lactose.

I_r and I_s are the intensity of radiation transmitted through the water reference sample and the milk sample, respectively. Equation (*1*) clearly illustrates the significance of the different interference mechanisms. The interference coefficient can be defined as follows (*8*) for interference on fat by protein and lactose, respectively:

$$(k_p P - k_{wf}\gamma)/(k_f P - k_{wf}\phi)$$

$$(k_l L - k_{wf}\delta)/(k_f F - k_{wf}\phi)$$

Analysis of these terms shows that the sign of the interference coefficient will depend on the strength of the absorption of the interfering component at the fat wavelength. Taking the protein interference as an example, for zero absorption ($k_p P = 0$) the interference will be negative, and, due to water displacement, the required correction coefficient will be positive. If the protein absorption is the more predominant of the 2 terms ($k_p P > k_{wf}\gamma$), the required correction will be negative. This explains the signs of the interference corrections in Table 1 where for the Fat A mode of operation, the interference corrections for protein and lactose are both positive values, which means that the net interference is predominantly due to water displacement; whereas in the Fat B and Fat (A + B) configurations, the net effects are negative, which means that the absorptions of both protein and lactose at 3.48 μm are the most significant mechanisms.

A similar equation can be derived for the single beam/dual wavelength or double beam/dual wavelength optical systems.

$$\ln(I_r/I_s) = K_1 F l - K_2\phi l + (K_3 P l - K_2\gamma l) + (K_4 L l - K_2\delta l) \ldots \quad (2)$$

Table 1. Typical values of correction coefficients for different calibration methods

Calibration method	Correction coefficients			
	X_1	X_2	X_3	X_4
F_A	0.98	0.03	0.07	—
F_B	—	−0.16	−0.24	0.98
F_{A+B}	0.27	−0.11	−0.16	0.73

where K_1, K_2, K_3, and K_4 are the algebraic differences between the extinction coefficients at the sample and reference wavelengths for fat, water, protein, and lactose, respectively.

K_1Fl and $K_2\phi l$ are the absorptions due to fat and water displacement by fat, respectively,

$K_3Pl - K_2\gamma l$ = net interference due to protein.

$K_4Ll - K_2\delta l$ = net interference due to lactose.

The interference coefficients can be defined in the same way as for the single beam system, but in this case twice as many individual extinction coefficients are involved. The same explanation for the sign of the corrections applies. It has been shown (6) that it is essential to make these corrections in order to obtain accurate results by the IR method. This correction procedure is the basic calibration of the IR instrument, but the manner in which it is achieved electronically varies from instrument to instrument.

A schematic diagram representing the correction circuitry of the Multispec M milk analyzer (Figure 8) will be used to help explain how the corrections are applied. The diagram represents 3-channel operation, namely fat, protein, and lactose, which is the same arrangement as the equations described previously. It can be seen that the fat signal, which is represented by $\ln(I_r/I_s)$ in equations (*1*) and (*2*), is first adjusted by the primary slope to give a measurement of the fat. This measurement still includes the interference from protein and lactose and so the corrections now have to be applied to cancel this error. The diagram shows that a fraction of the protein measurement, the protein on fat correction factor, and a fraction of the lactose signal, the lactose on fat correction factor, are taken into a summing junction along with a fraction of the fat measurement, after it has been modified by the secondary slope. The secondary slope provides an adjustment of the fat, which is independent of, and does not affect, the calibration of the protein and lactose channels. The diagram also shows that adjustment of any one of the primary slopes will modify the calibration of all 3 channels, one directly, and the other 2 channels through the correction factors. On some of the older generations of equipment, the circuitry does not include a secondary slope correction, which complicates the calibration procedure because adjustment of the primary slope effectively multiplies all the terms in equations (*1*) and (*2*) by a fixed amount. This means that the portion of the signal that results from the protein and lactose interference is also being adjusted, so the corrections coming from the protein and lactose signals no longer offset this new level of interference. In summary, therefore, adjustment of any one of the primary slopes either alters the magnitude of the corrections that are generated from the channel being adjusted, or upsets the balance between the interference from the minor components and their corrections from the other channels, which was previously calculated. For the same reason, the secondary slope should only be adjusted to insert the coefficients calculated by multiple linear regression. It should be noted that, if the adjustments to the primary slope are minor and are to correct for the changes in absorptivity that occur within the normal range for milk, the subsequent errors will be almost insignificant. Problems arise

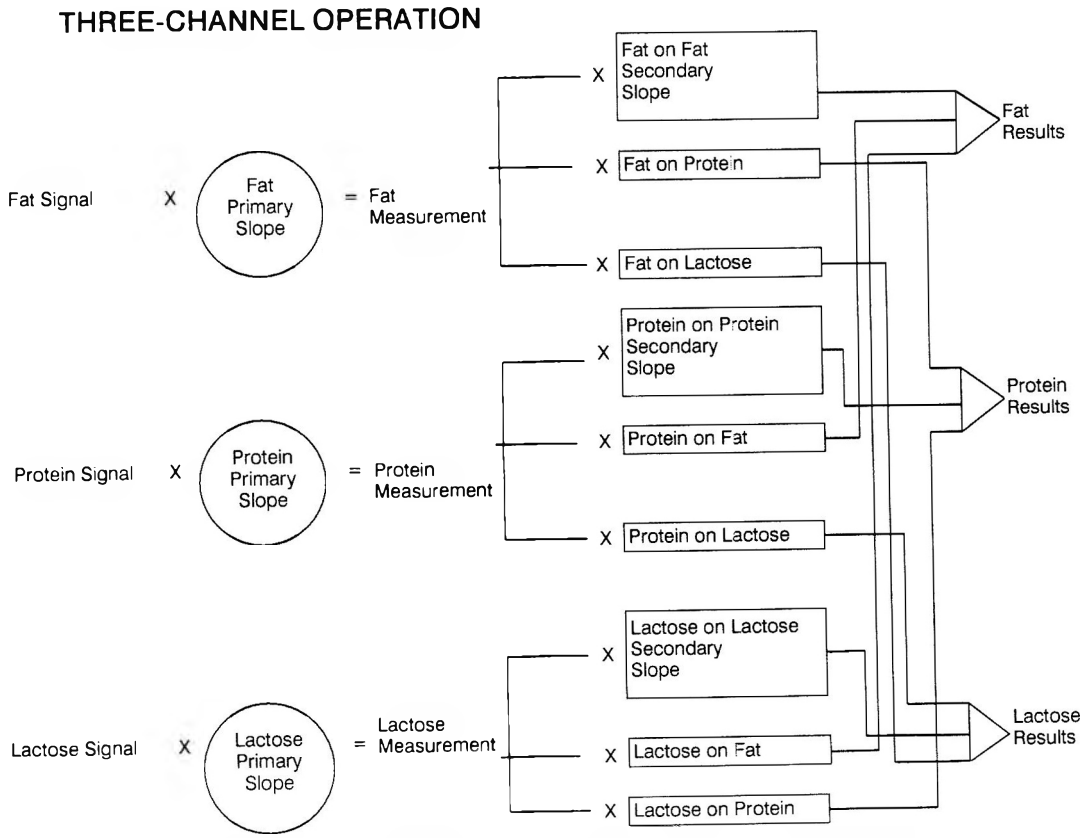

Figure 8. Diagram of the correction circuitry for a 3 channel milk analyzer.

if the adjustment is being made to compensate for instrument problems or environmental changes such as moisture in the optical system. The consequences in this case are that the accuracy of the instrument will be impaired. It is, therefore, important that the instrument is fully serviced and the optical compartment is kept dry.

Multiple linear regression (MLR) is now commonly used to calculate the interference correction factors and, hence, calibrate the instruments. To use this technique, it is necessary to collect instrument data before the corrections are applied. These data are referred to as uncorrected data and apply to the fat, protein, and lactose measurements in Figure 8. In some older models of equipment, which do not display uncorrected data, it is more difficult to obtain these measurements, and the corrections must be dialed to zero on the circuit boards so that the fat, protein, and lactose results are now uncorrected values for each channel. The solution to the regression calculations is described in the matrix of equations shown below:

$$F = X_1F_{unc} + X_2P_{unc} + X_3L_{unc} \ldots \quad (3)$$

$$P = X_4F_{unc} + X_5P_{unc} + X_6L_{unc} \ldots \quad (4)$$

$$L = X_7F_{unc} + X_8P_{unc} + X_9L_{unc} \ldots \quad (5)$$

$$TS \text{ (or SNF)} = X_{10}F_{unc} + X_{11}P_{unc} + X_{12}L_{unc} + K \ldots \quad (6)$$

where F_{unc}, P_{unc}, and L_{unc} are uncorrected results for fat, protein, and lactose, respectively; X_1 through X_{12} are regression coefficients, which have already been defined as interference correction factors; and K is the regression constant or intercept.

It can be seen that the equations for fat, protein, and lactose do not include a regression constant because the calculations have been modified to drive the calibration line through zero. The line can, therefore, be described as the line of best fit through zero and the data points, and is often referred to as zero regression or ZREG. This has proven to be a most useful technique and the advantages of pivoting the line through zero easily outweigh the need for an intercept. In the case of the main components, the only reason for a regression constant would be to account for the change in absorbance due to water displacement by the mineral content of the milk. This absorbance is relatively small and constant and its effect will be included in the regression coefficients of the main components. The regression constant in the solids calculation is intended to account for the mineral content, which cannot be measured directly using the infrared method, but is significant in measurement of the milk solids.

If the instrument is being used to measure the solids-not-fat (SNF) content of milk that has been heavily fortified with this component, a separate calibration line should be used which does not include an intercept. It can be shown that its presence will cause the modified SNF content to be underestimated because the result is not totally dependent on a proportional increase in absorption centers.

In newer instruments, the correction factors derived by the MLR calculations can be either dialed directly onto the scaling circuit boards or entered into the calibration section of the onboard computers. The coefficients X_1, X_5, and X_9 are the secondary slope values and the remaining coefficients are entered as correction factors.

In older instruments, which do not have a secondary slope control, MLR can still be used to set up the basic calibration, but because of the interaction of the primary slopes with the other correction factors in the circuit, these results have to be modified. The primary slope potentiometers now perform the X_1, X_5, and X_9 function and will require to be adjusted. For the reasons already discussed, the impact of this adjustment on the other correction factors has to be calculated and the final values determined. Equations (*7*), (*8*), and (*9*) have been derived to produce the final correction factors.

$$F = X_1F_{unc} + X_{13}P_{unc} + X_{14}L_{unc} \ldots \quad (7)$$

$$P = X_{15}F_{unc} + X_5P_{unc} + X_{16}L_{unc} \ldots \quad (8)$$

$$L = X_{17}F_{unc} + X_{18}P_{unc} + X_9L_{unc} \ldots \quad (9)$$

where $X_{13} = X_2/X_5$, $X_{14} = X_3/X_9$, $X_{15} = X_4/X_1$, $X_{16} = X_6/X_9$, $X_{17} = X_7/X_1$, $X_{18} = X_8/X_5$; and new fat potentiometer setting = X_1 × old fat potentiometer setting, new protein potentiometer setting = X_5 × old protein potentiometer setting, and new lactose potentiometer setting = X_9 × old lactose potentiometer setting. To obtain the uncorrected component values, the correction factors already installed on the circuit board must be dialed to zero.

Another method of applying MLR analysis with this type of instrument is to transmit the uncorrected signals to an external computer. Programs have been developed to perform the MLR calculations and create the calibration files. We believe that it is much easier to control calibration in software than by adjusting potentiometers.

Once the basic calibration has been developed, it must be maintained. One method that was suggested (A. J. Brand, California, Milk Producers, 1983, private communication) has proved to be extremely accurate: applying simple linear regression to the corrected results. This technique, which was named ABREG, can be used to fine tune the basic calibration and, using the fat channel as an example, can be described as follows:

$$F = a + b(X_1F_{unc} + X_2P_{unc} + X_3L_{unc}) \ldots \quad (10)$$

where a is the intercept and b is the slope factor. So far, for the sake of simplicity, the mathematical treatment has been limited to a 3-channel system. The idea of the $F_{(A+B)}$ mode of operation introduced in an earlier section can be defined as follows:

$$F = X_1F_{Aunc} + X_2P_{unc} + X_3L_{unc} + X_4F_{B\,unc} \ldots \quad (11)$$

and with ABREG

$$F = a + b(X_1F_{A\,unc} + X_2P_{unc} + X_3L_{unc} + X_4F_{B\,unc}) \ldots \quad (12)$$

The inclusion of F_B as the fourth term in the equation allows an extra degree of freedom, which is the source of more variability in the basic calibration. For this reason, we decided to extend the study to investigate the potential of the F_A proportional F_B method of operation (APB), which was also referred to in an earlier section. The equation for this method can be written as follows:

$$F = 0.27F_A + 0.73F_B \ldots \quad (13)$$

and with ABREG

$$F = a + b(0.27F_A + 0.73F_B) \ldots \quad (14)$$

where

$$F_A = X_1F_{A\,unc} + X_2P_{unc} + X_3L_{unc} \ldots \quad (15)$$

$$F_B = X_4F_{B\,unc} + X_{2*}P_{unc} - X_{3*}L_{unc} \ldots \quad (16)$$

X_{2*} and X_{3*} are correction coefficients for a 3-channel system using F_B in place of F_A.

Table 2. Mean and standard deviation of differences between instrument estimates and standard reference results using multiple regression for calibration calculations: data for fat, protein, and solids-not-fat obtained on 3 instruments

	Fat			Protein			SNF		
Statistic	1	2	3	1	2	3	1	2	3
No. of samples:	500	449	370	463	437	343	475	444	347
Mean diff.:									
A	0.009	0.001	0.004	0.008	0.000	0.000	0.026	0.002	0.012
B	0.003	−0.007	0.003	0.008	0.000	−0.001	0.026	0.001	0.011
APB	0.004	−0.005	0.003	0.008	0.000	0.000	0.026	0.001	0.011
(A + B)	0.004	−0.005	0.004	0.010	0.004	0.000	0.025	0.004	0.015
Std dev. of diff.:									
A	0.040	0.038	0.041	0.054	0.052	0.049	0.060	0.056	0.066
B	0.031	0.035	0.035	0.054	0.052	0.050	0.060	0.056	0.066
APB	0.028	0.032	0.031	0.054	0.052	0.050	0.060	0.056	0.066
(A + B)	0.029	0.032	0.034	0.055	0.054	0.053	0.062	0.054	0.069

A comparison of the accuracy of the different methods of operation is shown in Tables 2 and 3, and is discussed in the results section.

Method Evaluation

Data for this study were submitted by 2 laboratories in California and one laboratory in Ontario, Canada. Results from the California laboratories were uncorrected signal data obtained by each laboratory with calibration samples provided by the California Department of Agriculture, who also supplied the standard reference method results. Fat calibration equations for the A, B, and (A + B) method of operation were generated. Equations were also constructed for the protein, lactose, and SNF components using the A wavelength from the signal and standard reference method data, and were then used to calculate estimates for the same samples. As a consequence, the estimates do not represent analysis on unknown samples with respect to mean difference, but with respect to standard deviation of difference are estimates of the best results obtainable with the milk samples and instruments used. They demonstrate also that with close control of calibration parameters (which was used in both laboratories), one can expect reasonably low mean difference and standard deviations of difference well within accepted limits for the method. However, to demonstrate this conclusively, one must have data on randomly chosen samples that were not included in the samples used to calculate the relevant calibrations.

The data from the Guelph laboratory was calculated similarly from data for 3 different instruments using different randomly chosen samples from the production of each instrument on a daily basis over a 5 month period. All signals were adjusted for purge correction and measured signal drift as monitored by control milks. Calibration equations were recalculated on a weekly basis. All estimates were obtained on random samples selected for the week following a calibration and represent unknowns. The data represent the results that would have been obtained with the various calibration types on unknowns selected at random from a population of over 10 000 herd milks in the province of Ontario. Quality control adjustments (by comparing daily mean differences with difference limits) purposely were not used so as not to interfere with the comparison of calibration types. With quality control adjustments, the accumulated mean differences for the whole period would have been smaller. The fact that standard deviations of difference were comparable to those obtained with the California data proves that, with optimum control of calibration parameters, the optimum expectations of the method can be realized.

For the Ontario data, accumulated statistics were compared for calibrations using data for 10, 20, and 50 calibration samples. For each of these, the calibrations were recalculated at intervals of 1, 2, and 4 weeks. Standard reference results were obtained using the Mojonnier method for fat, a semimicro Kjeldahl method for protein (**16.036**, using 0.5 g sample with reagent amounts proportionally adjusted), and the AOAC oven-drying method (**16.032**) for total solids. The calibrations were for fat, protein, and solids-not-fat. The latter chemical result is calculated by subtracting fat from total solids. Calibration equations were calculated using a multiple regression model with a zero-based origin. Following an original calibration, the instructions for some instru-

Table 3. Mean and standard deviation of differences between instrument estimates and standard reference results using simple regression on estimates with original equations: data for fat, protein, and solids-not-fat obtained on 3 instruments

	Fat			Protein			SNF		
Statistic	1	2	3	1	2	3	1	2	3
No. of samples:	500	449	370	463	437	343	475	444	347
Mean diff.:									
A	0.013	0.003	0.009	0.005	−0.002	0.000	0.023	0.003	0.018
B	0.003	−0.005	0.004	0.004	−0.002	0.000	0.021	0.002	0.017
APB	0.006	−0.003	0.005	0.005	−0.002	0.000	0.022	0.002	0.017
Std dev. of diff.:									
A	0.038	0.037	0.040	0.050	0.051	0.052	0.059	0.050	0.066
B	0.028	0.035	0.029	0.050	0.051	0.052	0.059	0.050	0.066
APB	0.026	0.032	0.027	0.050	0.051	0.052	0.059	0.050	0.066

ments involve using the equations as constants. Calibration is then adjusted with simple regression calculations, using chemical reference values as the dependent variable and estimates as the independent variable (ABREG). The new estimates are then obtained by applying the intercept and regression coefficient to the estimates from the original equation. To assess the effectiveness of this method for updating calibrations, it was applied to the data obtained with 20 samples used for original calibration with a 1-week period between successive calibrations.

Results

For the comparisons between estimates and reference results, the combination that gave the best overall results in the Canadian study was that which used data for the last 20 samples in the calibration databank for calculating equations and a period of 1 week for application of these equations. Mean differences and standard deviations of difference for this combination are shown in Table 2.

The AOAC specifications for these standard deviations are ≤0.06% for fat or protein and ≤0.12% for total solids. Although in this study the solids determination was for SNF, the estimates all are well within expectations for the method. For fat, the APB type calibration gave the smallest overall standard deviation; with the (A + B) and B method, standard deviations only slightly larger. Standard deviations for the A method, although well within specification, were larger in every case. For protein and SNF, no standard deviations were significantly different for the different calibration types.

The mean differences in Table 2 are all well below the AOAC specifications of ≤0.05% for fat or protein and ≤0.09% for total solids. However, the AOAC specifications are for results for only 8 samples. With the larger numbers of samples involved in this study, some of the mean differences, particularly those for SNF with instruments 1 and 3, are outside statistical limits. This can be avoided if normal quality control procedures are used to control the accumulated mean differences. Corrective adjustments are made to the intercept of a simple regression equation used outside the main equation whenever the mean difference for a single day exceeds its statistical limit or whenever 6 or more successive daily mean differences are all on the same side of zero. Although it is common practice to make such adjustments in the laboratory using this method, the authors did not wish to interfere in this way with the comparison between calibration types, and no such adjustments have been made.

From the data in Table 2, the conclusion can be drawn that the newer infrared analytical methods, which use absorptions by the CH_2 and CH_3 groups in the fatty acid chain of fat molecules either instead of or in addition to absorptions of the carbonyl groups, are somewhat better than the present AOAC method and should be adopted by AOAC.

Comparison data for the calibration methods, which update calibration equations by using simple regression calculations on a constant main equation, are shown in Table 3 for three of the calibration types. Most of the standard deviations are slightly smaller than those in Table 2. Mean differences were sometimes larger and sometimes smaller. However, one can conclude that this method for adjusting calibrations is equally as good as that used to generate the data in Table 2.

The results obtained for the test data collected from the 2 laboratories in California are shown in Table 4. The standard deviations of difference for the different analytical methods are all well within the specifications required by AOAC. A comparison of the accuracies for the different methods supports the conclusions derived from the Canadian study.

Recommendation

It has been demonstrated that the measurement of infrared energy absorptions by CH_2 and CH_3 groups in the fatty acid chains of fat molecules either instead of or in addition to measurements of absorptions by carbonyl groups improves the accuracy of the analysis relative to that for AOAC method **16.083–16.092**. It is recommended that the method for infrared analysis of milk be revised to include the use of absorptions at 3.48 μm, with additions to sections **16.083, 16.085, 16.086, 16.088, 16.090, 16.092,** and additional sections to apply when a computer is used for calibrations and quality control.

As a result of the interlaboratory evaluation, the following revisions and additions are recommended:

(a) Change the first line of the title to read:

Fat, Protein, Lactose, and Solids in Milk

(b) Change **16.083,** *Principle*, to read:

Analysis of milk by IR is based on absorption of IR energy at specific wavelengths by CH groups in fatty acid chain of fat molecules (3.48 μm), by carbonyl groups in ester linkages of fat molecules (5.723 μm), by peptide linkages between amino acids of protein molecules (6.465 μm), and by OH groups in lactose molecules (9.610 μm). Total solids (TS) or solids-not-fat (SNF) are computed by assigning experimentally detd factor to percentage of all other solid milk components, and by adding this amt to appropriate % fat, protein, and lactose, or by direct multiple regression calcns using instrument signals at combinations of above-mentioned wavelengths. Latter method has been shown to be more accurate method of detg milk solids. Analysis by IR is dependent on calibration against suitable std method. See "Definitions of Terms and Explanatory Notes," item (24), for calcn of regression lines.

(c) Add as a second paragraph in **16.085:**

Instrument must be well maintained and functioning correctly. Malfunctions that influence calibration can cause large errors.

(d) Add as a second calibration procedure in **16.086** and **16.088:**

Alternatively, for better accuracy, use simple linear regression equation that relates instrument ests and std ref. values, using latter as dependent variable, to correct these ests. In earlier, instruments, which do not have this capability in instrument software, this calcn, must be performed outside instrument. To perform this calcn on calibration data that are collected on successive days or weeks, it is necessary to

Table 4. Evaluation of test data collected from 2 California laboratories in interlaboratory evaluation of IR method for fat analysis in milk

Statistic	F_A	F_B	F_{A+B}	P	L	SNF
Laboratory 1:						
Std dev.						
(46 samples)	0.035	0.033	0.029	0.04	0.06	0.06
Method bias	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Laboratory 2:						
Std dev.						
(46 samples)	0.04	0.031	0.026	0.04	0.06	0.05
Method bias	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01

record instrument ests as well as final results. Purpose of this alternative, preferred procedure is to avoid adjusting instrument slope controls.

(e) Add as a second calibration procedure in **16.090:**

Alternatively, for better accuracy, multiply instrument ests by $\Sigma L'/\Sigma L$ to obtain final results. In earlier instruments, which do not have this capability in instrument software, this calcn must be performed outside instrument. To perform this calcn on calibration data that are collected on successive days or weeks, it is necessary to record instrument ests as well as final results. Purpose of this alternative, preferred procedure is to avoid adjusting instrument slope controls.

(f) Add as a second calibration procedure in **16.092:**

Alternatively, calibrate by multiple regression to calc. equation for estn of either TS or SNF as function of fat, protein, and lactose uncorrected signals. For recalibration, use simple linear regression equation which relates regression ests and std ref. values, using latter as dependent variable, to correct these ests and obtain final result. In earlier instruments, which do not have this capability in instrument software, this calcn must be performed outside instrument. To perform this calcn on calibration data that are collected on successive days or weeks, it is necessary to record regression ests as well as final result. This alternative, preferred method has been demonstrated to be more accurate.

Note: For products that are fortified or dild, both original multiple regression and simple regression calcns must be based on regression that forces calibration line thru origin. First calcn procedure in this sec. (**16.092,** par. 1) cannot be used successfully with these products.

(g) Add new sections to apply when a computer is used for calibration and quality control:

16.E01 ***Computer-Based Calibration and Control***

Program computer to:

(*1*) Calc. $S_2' = S_1 + [(S_2 - S_1)/PF]$, where S_2' = purge-corrected signal; S_2 is signal at any instrument channel; S_1 is same signal for previous sample; and PF is predicted purge fraction = purging efficiency/100 (see **16.085**). Apply correction to all signals received from instrument.

(*2*) Calc. $(\Sigma CS_2')/10$, where CS_2' = purge-corrected control milk signal. Sum control milk signals at each channel for 10 control samples, and average. At time of calibration, store and designate as required control milk signals.

(*3*) Calc. $S = S_2' + CSE$, where S = purge- and drift-corrected signal; CSE is control signal error = (required control signal av.) − (detd control signal av.) for any check on control milk. Apply value to all except control milks.

(*4*) Calc. ests from purge- and drift-corrected signals using calibration signals, and store. Equation type is Est = A + B (main equation). Initially, A = 0 and B = 1.

(*5*) Calc. calibration equations and store.

(*6*) Store random sample signals, ests, and std method ref. results.

(*7*) Calc. and store mean difference, std dev. of difference, limits for mean difference, and population std dev. of difference for statistical quality control.

(*8*) Store results for checks on purging efficiency tests, homogenization efficiency tests, linearity tests, and repeatability tests.

16.E02 ***Instrument Controls***

For instrument with secondary slope controls, operate in uncorrected mode. For instruments without secondary slope controls, reduce interference correction coefficients to 0.

16.E03 ***Control Milk***

Prep. adequate supply of pasteurized, homogenized, preserved milks with % fat about that of population av. Transfer amts for single tests to sample containers and keep refrigerated. Zero-adjust instrument channels. Heat 11 samples to 40°, and pump each thru instrument, collecting signals. Average last 10 results for each signal to calc. required signal. Record required control milk signals to 4 decimals to prevent rounding errors in continuous control situation.

16.E04 ***Setting Primary Slope Controls***

Det. % fat, protein, and lactose on series of ≥8 preanalyzed (e.g., **16.064, 16.036, 16.055,** or **16.059**) milks of type to be analyzed by instrument. Warm samples to 40°, mix thoroly, and pump thru instrument, collecting and correcting signals for purge and drift. For each signal, calc. regression equation of type $S = aF + bP + cL$, where S is signal and *F*, *P*, and *L* are results for std. At fat channel, adjust slope amplification by 1/a. At protein and lactose channels, adjust by 1/b and 1/c, resp. Check success of attempt to obtain coefficient of 1 for main components by repeating test. Readjust slope amplifications if necessary. Lock slope controls and do not change settings thereafter. Warm 11 samples of control milk and redet. required control milk values as indicated above.

16.E05 ***First Calibration***

Obtain 20 milk samples by random selection from population to be tested, and analyze by std ref. method for components to be estd by IR method. Key std results into computer. Warm and prep. 11 control milk samples and the calibration samples. Pump thru instrument and collect and store signals. Computer applies purge correction, calcs control signal correction values, applies these to calc. milk signals, and stores these values in matrix with std ref. values. Select equation that is applicable to instrument from following main equation types.

Components	*Signals*			
	F_A	F	L	F_B
1. F, P, L, TS, SNF	*	*	*	
2. F, P, L, TS, SNF		*	*	*
3. F	*	*	*	*
4. F	$[0.27 \times F(1)] + [0.73 \times F(2)]$			

Note: Except for TS and SNF, all main equation types force regression line thru 0. With TS and SNF, either may be used, but equation with intercept is preferred and gives better results unless product being analyzed has been fortified or dild.

Calc. by multiple linear regression main equations for components for which IR analysis is required. It is not necessary to calc. ests for any component for which ests are not required or to perform std ref. method analysis for that component except when slopes for initial calibration are adjusted. However, all signals must be controlled using required control milk signal values.

16.E06 ***Production Analysis***

At beginning of each production period and at definite intervals during production run, check control milk signals for minimum of 3 samples and recalc. drift corrections for application to product samples. Interval between checks can be shortened if drift for current interval is excessive. If instrument signals stability is acceptable, 1-h interval is satisfac-

tory. Signals for product samples are corrected for purge and drift and then are used to calc. ests.

16.E07 ***Quality Control***

Select production samples at random at predetd intervals and perform std ref. method analyses on these. Store signals, ests, and ref. values for these samples in matrix on accumulative basis. Check control milks and make adjustments to drift correction just prior to these selections. Interval between selection (days) and number selected may be varied to suit laboratory but ≥5 should be selected in 1 day and ≥20 in 1 week. Current population std devs of difference between est. and ref. methods may be calcd from last 50 differences in these accumulated data.

For quality control, calc. statistical limits for daily mean difference as 3 × population std dev./$\sqrt{N}$, where N is number of random samples tested that day. Action situations for changing A intercept in overall equations are:

(*1*) Daily mean difference is greater than limit.

(*2*) Six successive or 7 of 8 successive or 8 of 10 successive mean differences are on same side of 0. To correct this, adjust A in opposite direction to mean of differences. To correct for (*1*), adjust A in opposite direction by excess of difference over limit plus one-third of limit. Suggested addnl statistical calcns are: (a) accumulated monthly mean difference, std dev. of difference, and limits for mean difference; and (b) accumulated annual mean difference, std dev. of difference, and limits for mean difference. Overall objectives are to control current analyses as well as possible and to achieve annual mean difference very close to 0.

16.E08 ***Recalibration***

Use signals and std ref. values for last N samples in accumulated random sample data. Calcn may be made either to redet. coefficients of main equation or by simple regression using ests of main equation as independent variable and std ref. method values as dependent variable. Latter method would det. values for A and B in overall equation.

Whenever control milk is changed, whether at time of recalibration or between calibrations, run 11 samples of both old and new control milks. Use drift errors of old control milk to adjust avs for new control milk signals in calcg required signals for it. Do not use control milk for longer than 1 week.

With random sample data accumulated over long periods of time, number of samples used for calibration and frequency of calibration can be optimized. Signals and std ref. values in data bank can be used to vary choices and det. net effect for different equations generated in this way.

For test population used first with this control method, optimum conditions were N = 20 and interval between calibrations = 1 week.

16.E09 ***Precautions***

See **16.085** for normal precautions. This control method uses required control milk signals to control calibration level in preference to maintaining 0 settings at various channels with H_2O. Advantages are that drift errors identify signal subject to drift and that control of level is in range of test analysis. Water zeros may still be checked. Drift away from 0 indicates extraneous absorptions which could be due to excessive moisture in console, buildup of scale on cell windows, or other instrument faults. Continuous drifting in 1 direction for 0 signal should be investigated.

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DRUG RESIDUES IN ANIMAL TISSUES

Thin-Layer Chromatography/Bioautography Method for Detection of Monensin in Poultry Tissues

PERRY A. VANDERKOP
Toxicology Research Group, University of Saskatchewan, Saskatoon, Saskatchewan S7N 0W0, Canada
JAMES D. MACNEIL[1]
Health of Animals Laboratory, Agriculture Canada, 116 Veterinary Rd. Saskatoon, Saskatchewan S7N 2R3, Canada

Monensin is an effective anticoccidial agent widely used in the poultry industry. Because of concerns over its toxicity, a sensitive, reliable, fast, and simple method for residue detection in poultry tissues is desirable from both a diagnostic and a regulatory view. Many methods of detection are excluded due to this ionophore's complex chemical and biological nature. A common method used for its detection is thin-layer chromatography/bioautography (TLC/B), although this is usually only semiquantitative. A new TLC/B method for monensin detection in poultry tissues was developed and is reported here. Recovery from cardiac muscle, skeletal muscle, and liver and kidney tissues is in the range of 93–97%. A detection limit of 250 μg/kg with 99% reliability was achieved with this method. Lower limits (to 10 μg/kg) were detectable, but with lower reliability (60%). Quantitative analysis is not possible on samples from fatty tissue.

Monensin is a monocarboxylic acid ionophore (1). It is produced by the bacterial order *Actinomycetales*, in the family *Streptomyces* (2). Methods of analysis are limited because of its complex chemical and biological properties. Monensin is nonvolatile, has no ultraviolet absorption above 220 nm, has a high molecular weight, and has multiple functional groups, including a spiroketal which makes the molecule acid-sensitive (2, 3).

Methods that have been used for the detection of monensin include liquid chromatography (LC), with detection via the formation of a fluorescent derivative (3–5), LC analysis using a refractive index detector (6), thin-layer chromatographic separation with detection by fast atom bombardment mass spectrometry (7), modified indirect enzyme-linked immunosorbent assay (8), colorimetry (9), photometric microbiological assay (10), and thin-layer chromatography/bioautography (11, 12). The latter method has proven to be the simplest and most popular method for detection of monensin in biological sample matrixes.

Several variations of the thin-layer chromatography/bioautography procedure have been described, using a variety of solvent systems and experimental conditions. Several of these procedures are only semiquantitative, and claims of high recovery and low detection limits have proven to be unfounded. For example, the Analytical Methods Committee (13) studied the methods of Okada et al. (12) and Tihova and Peneva (14) and observed that these methods were only semiquantitative at levels below 0.1 mg/kg, and that the detection limits and recovery values claimed by the original authors could not be replicated (13). This was also observed in the present study when the procedures of Donoho and Kline (11), Martinez and Shimoda (15), and Neidert et al. (16) were applied to a variety of poultry tissue samples (17).

A modified thin-layer chromatography/bioautography method was therefore developed, based on previous investigations (11, 16). This method meets time requirements for regulatory residue methodology, is simple to perform, and is highly reliable for the detection of monensin residues extracted from a variety of poultry organ tissue matrixes.

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[1] Address correspondence to this author.

Experimental

Apparatus and Reagents

(a) *Centrifuge tubes.*—Polyethylene, 50 mL tube with screw cap (Falcon Blue Max) from Baxter/AHS, Edmonton, Alberta, Canada.

(b) *Silanizing reagent.*—AquaSil (Pierce Chemical Co.), from Chromatographic Specialties Co., Brockville, Ontario, Canada.

(c) *Homogenizer probe.*—Kinematica, Model PT-10-35.

(d) *Mechanical shaker.*—Eberbach flask shaker, Model 6000.

(e) *Centrifuge.*—IEC International Centrifuge, International-Universal Model UV.

(f) *Rotary evaporator.*—Buchi Rotavapor R-110.

(g) *Conical centrifuge tubes.*—15 mL, Pyrex.

(h) *Analytical evaporator.*—Meyer N-Evap, Model 112, Organomation Associates, MA.

(i) *TLC plates.*—Whatman KL6D silica gel, from Chromatographic Specialties Co.

(j) *Spotting syringe.*—Hamilton, 50 μL glass syringe, from Chromatographic Specialties Co.

(k) *Heating strip.*—84 cm × 1 cm, made in laboratory.

(l) *Developing tank.*—Glass, 9 cm × 26 cm × 29.5 cm, lined with filter paper.

(m) *Solvents.*—All solvents distilled in glass, Omnisolv grade, BDH.

(n) *Standard.*—Monensin sodium salt, from Sigma Chemical Co., St. Louis, MO.

(o) *Bioassay plates.*—Sterilized plastic, 24.3 cm × 24.3 cm × 1.8 cm (Nunc), from Gibco, Burlington, Ontario, Canada.

(p) *Assay organism.*—*Bacillus subtilis* ATCC 6633 Difco spore solution, 1 mL ampoules, from Baxter/AHS, Toronto, Ontario, Canada.

(q) *Autoclave.*—Consolidated Stills and Sterilizers, Boston, MA.

(r) *Incubating oven.*—Fisher, Isotemp oven, 300 series.

(s) *Bioautographic media reagents.*—Difco certified, from Baxter/AHS.

(t) *Triple salt solution agents.*—Reagent grade, Fisher.

(u) *Citric acid, sodium hydroxide.*—Reagent grade, Fisher.

(v) *TZC solution.*—Reagent grade 2, 3, 5-triphenyltetrazolium chloride (BDH), dissolved in water to 0.2%.

(w) *Sodium phosphate.*—Anhydrous (J. T. Baker), from Johns Scientific, Toronto, Ontario, Canada.

Preparation of Tissue Extracts

All experimental glassware was silanized before use to increase the recovery of the monensin. Ten g diced, frozen

tissue was placed into a 50 mL polyethylene centrifuge tube. Twenty mL methanol was added and the contents were homogenized for 20 s using a Polytron probe. The probe was rinsed twice with 2.5 mL methanol, and the rinse was added to the contents of the centrifuge tube. The cap of the centrifuge tube was tightened, and the tube was placed on a mechanical shaker and shaken for 15 min at a low setting. The tube was then centrifuged for 10 min at 2200 rpm. The supernatant liquid was transferred to a 150 mL separatory funnel using a small Pasteur pipet and extracted with three 30 mL volumes of carbon tetrachloride. The carbon tetrachloride extracts were combined in a 200 mL round-bottom flask and evaporated to dryness on a rotary evaporator set at 50°C. The round-bottom flask was rinsed twice with 4 mL hexane, and the combined rinses were collected in a 15 mL conical glass centrifuge tube. The sample was evaporated to dryness using a nitrogen-flow evaporator set at 60°C. The residue was solubilized in 1.0 mL methanol.

Thin-Layer Chromatography

The absorbant zone of a Whatman LK6D silica gel TLC plate was preheated with a heating strip attached to a variable autotransformer, set to provide a temperature of 20°C. Using a micro-syringe, 30 μL extract was spotted on the preheated TLC plate and allowed to dry for ½ h. The plate was developed for 45 min in a sealed glass tank containing a mixture of chloroform–methanol–acetone–glycerol (98 + 60 + 40 + 2), that had been allowed to equilibrate at least 2 h, as reported by Neidert et al. (16). The TLC plate was removed from the chamber and the solvent front was marked immediately with a pencil. The TLC plate was dried in the air for 2 h in a fume hood.

Bioautography

The plating medium was prepared as reported by Salisbury et al. (18) by adding 1.5 g beef extract, 3.0 g yeast extract, 4.0 g tryptone, 6.0 g peptone, and 1.0 g dextrose to 1 L distilled water. Five mL triple salt solution (magnesium chloride, manganese sulfide, calcium chloride), followed by 2.1 g citric acid and 4.7 g sodium phosphate, were added to the plating medium. The plating medium was adjusted to pH 5.5 by adding either 0.1M citric acid or 0.1N sodium hydroxide. One hundred mL medium was dispensed into 250 mL Erlenmeyer flasks containing 1.0 g Noble agar. The flasks were autoclaved 20 min at 121°C and mixed well as they were removed from the autoclave.

After cooling, the plating medium was remelted by immersing the flask in boiling water for 20 min. It was then removed from the water bath, let to cool to 50°C for 10 min, and 0.6 mL *Bacillus subtilis* spore suspension was added to the medium; the flask was swirled carefully for 30 s to minimize formation of bubbles. The seeded medium was poured into a sterilized Nunc plastic bioassay dish, and allowed to cool and harden for 30 min. The developed TLC plate was then placed face-down on the bioassay dish for 12 min. The TLC plate was then removed and discarded. The bioassay dish was inverted and incubated at 37°C for 14 h after which it was removed from the incubator and the medium surface was sprayed with the tetrazolium chloride (TZC) solution. The dish was then re-incubated for 25 min. The bioautogram was either photographed or photocopied and the area of the zone of inhibition was measured.

Results and Discussion

Optimization of Procedure

The procedure conditions were optimized to enhance monensin detection in poultry tissue samples. Four tissues were studied: cardiac muscle, skeletal muscle, liver, and kidney. In our experience, these are representative of tissues made available to the analyst in toxicological investigations. To maximize the amount of monensin that could be recovered from the sample tissues, the effects of glassware silylation, centrifuge speed, and extraction solvents were studied. TLC solvent developing systems, media, media pH levels, and incubation periods were also studied to improve the detectability of monensin during bioautographic plate development.

Although assay of the muscle, liver, and/or kidney tissues will not necessarily give a true indication of the monensin status of the bird (1, 11), it will nevertheless yield quantitative information on the monensin concentration in edible tissues. Furthermore, the presence of significant monensin residues in these tissues would be a useful indicator of monensin toxicosis in a poultry flock. The procedure as listed was tested and found to be unsuitable for the analysis of fatty tissue, because zones of inhibition produced with such extracts were irregular and not quantifiable. Since a different extraction and purification scheme would have had to been established for fatty tissues, it was decided that this procedure would be developed to deal strictly with analysis in the above-listed tissues. It has been found in this laboratory that the method of Okada et al. (12) is useful for detection of monensin in fatty tissue, although it is only semiquantitative below 0.1 mg/kg (13).

Silylation of glassware before extraction increased the amount of monensin recoverable in all of the tissue types by approximately 10%, from an average of 81.3% to 94.5%. This indicates that some binding of monensin to laboratory glassware occurs, and silylation is therefore recommended to remove the active sites, thus allowing monensin to partition into the solvent system.

The optimal centrifuge speed was 2200 rpm, although the improvement was of a lower magnitude than occurred with silylation. The improvement was in the range of 5% over centrifugation speeds ranging from 1800 to 2800 rpm. It appears that 2200 rpm is the best speed for the partitioning of monensin into methanol; it is in the middle of the centrifugation range studied, and 5% improvement can be considered to be within experimental error.

Different solvent systems were also studied. The methanol–carbon tetrachloride system (11) proved superior to the other 2 systems tried. Recoveries among tissues ranged from 93 to 97%. The methanol–chloroform system (16) had considerably lower recoveries, in the range of 62–67%, about 30% lower than the former system. The methanol–water–methylene chloride system (15) produced the lowest recoveries.

The chloroform–methanol–acetone–glycerol developing system enabled a detection limit of 100 μg/kg to be achieved (Table 1). This was roughly 1/7 of the limit of the carbon tetrachloride–benzene–methyl cellosolve system, with detection limits of only 750 μg/kg, and 1/5 of the limit of the ethyl acetate–acetonitrile system, with detection limits of 500 mg/kg. The methanol–water and methylene chloride–methanol plate developing systems were not suitable, having much poorer detection results. The added sensitivity observed for the solvent systems containing glycerol and cellosolve was attributed to residual quantities of these high boiling components of the solvent mixtures remaining on the TLC plate after drying, thereby providing monensin in solution instead of absorbed on the TLC layer-material and thus promoting its diffusion into the bioassay medium.

Medium pH and incubation period were found to affect the size of the zone of inhibition produced by the monensin on

Table 1. Effect of different plate developing systems on monensin detection limit[a]

System solvents	Detection limit, mg/kg
Chloroform–methanol–acetone–glycerol (98 + 60 + 40 + 2)	0.100
Methanol–water (80 + 20)	10.000
Methylene chloride–methanol (90 + 10)	10.000
Carbon tetrachloride–benzene–methyl cellosolve (80 + 10 + 5)	0.750
Ethyl acetate–acetonitrile (50 + 50)	0.500

[a] Results of 3 replications.

the bioautographic plate. The largest zones and highest correlations were produced with the pH 5.5 bioautographic medium. When the pH fell below 4.0, no zone of inhibition was produced. When pH levels were above 7.0, only moderate success was achieved when large concentrations of monensin were present. This is a result of the optimal environmental conditions for bacterial spore growth; too acidic or basic media resulted in a decline in spore growth.

The optimal incubation period for the bioautographic plates was 14 h. Time periods longer than 14 h produced smaller zones of inhibition, perhaps due to overgrowth by the bacterial spores. Time periods under 14 h yielded plates with a "washed-out" appearance, and quantitation was not possible.

Quantitation of Procedure

The area of the zone of inhibition correlated (r = 0.980) with the concentration of monensin present in the tissue sample (Table 2), making this procedure quantitative (Figure 1). Since the zones of inhibition produced were circular, the zonal size was measured by placing a pre-drawn clear cross over the zone in order to determine the mid-point of the circle (that point at which each arm of the cross measured equal distance from the zone center) and the measurement of the radius. Once the radius was measured, the total area could be calculated. This procedure is also highly reliable, 99% at 20 replications, to 100 μg/kg. However, due to the small zone size at this concentration, and the difficulty involved in its measurement, a detection limit of 250 μg/kg may be a more appropriate value for diagnostic laboratories, because the zone size is large enough to measure readily. Levels as low as 10 μg/kg can be detected, although the level of reliability decreases to 60%. Although the high reliability

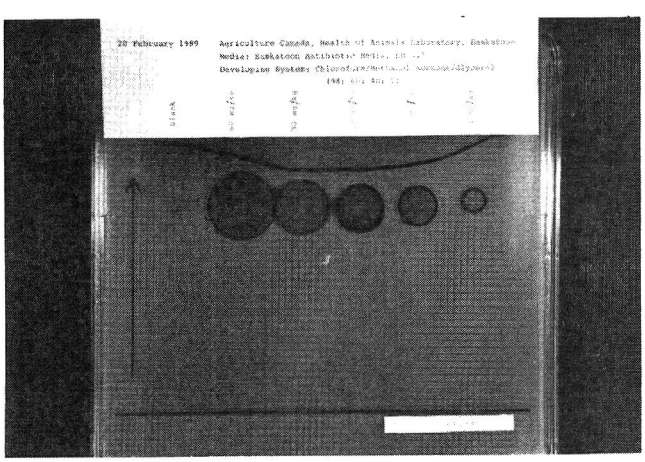

Figure 1. Typical bioautogram of monensin showing varying concentrations.

Table 2. Tissue monensin concentration related to size of zone of bacterial inhibition[a]

Monensin level	Zone of inhibition,[b] sq. mm (± SD)
100 mg/kg	389.0 ± 0.01
50	257.0 ± 0.01
25	162.2 ± 0.01
10	97.7 ± 0.01
1	22.9 ± 0.02
750 μg/kg	20.9 ± 0.01
500	15.5 ± 0.00
250	10.0 ± 0.01
100	5.5 ± 0.02
50	3.6 ± 0.01
25	2.4 ± 0.01
10	1.4 ± 0.01

[a] Correlation coefficient, r = 0.980.
[b] Results of 20 replications.

and high correlation of the zone of inhibition to the concentration of monensin in the sample could enable sample analysis to be completed without a range of monensin standards on the same plate, it is still advisable to run a range of standards on each plate, ensuring a standard response is achieved with each analysis.

Since this method uses a microbial organism for monensin detection, there was speculation that the method may have been subject to error in terms of false positive results arising from nonspecific inhibition. Colonies of a variety of bacterial spoilage organisms have been experimentally demonstrated to increase exponentially in chicken liver and muscle samples following bird death (19), and lead to nonspecific inhibition with microbiological screening tests for antibiotics. The susceptibility of attaining false positives increases with extraction and concentration procedures designed to improve method sensitivity (19). However, it has been the experience in the authors' laboratory that these nonspecific inhibitory substances migrate at different rates than most antibiotics, leading to a separation evident on the TLC plate by measurement of the R_f value, and also readily identifiable upon bioautography.

It was found that in this procedure both lasalocid and salinomycin interfered with monensin, all 3 having an R_f value of 0.80 (in the chloroform–methanol–acetone–glycerol developing system). Fortunately, lasalocid can be specifically identified by the characteristic "teardrop" shape of its zone of inhibition on bioautographic media, distinguishing it from monensin and salinomycin which have characteristically round zones. These 3 ionophores can be separated from a tissue sample by using the extraction method described, spotting 30 μL extract on a TLC plate, and developing the plate in a glass tank containing a mixture of ethyl acetate and acetonitrile (50 + 50) for 35 min, followed by the bioautography as described. The R_f values for monensin, lasalocid, and salinomycin are 0.12, 0.34, and 0.21, respectively. With this solvent system, all 3 ionophores retain their characteristic zonal shape. While the method as described in this paper is therefore suitable to use to analyze samples specifically for monensin, prudence suggests running a preliminary screening plate using the ethyl acetate–acetonitrile (50 + 50) solvent system if there is reason to suspect the presence of other ionophores.

The procedure as listed here is the most sensitive and reliable one for monensin residue detection in poultry tissue samples reported to date. Due to differences in the matrix composition of tissues from other animal species, this procedure may not be suitable for the detection of monensin in

those other species which regularly consume monensin in their feed, notably cattle and swine. Plating results, such as the shape and size of the zone of inhibition, are also known to differ as the amount and nature of the impurities in an extract change (11), although the actual R_f value may remain the same. It was a fortuitous occurrence that the zone of inhibition produced with poultry tissue extracts was circular. However, this may not necessarily be the case in tissue samples from other species. Zones of inhibition produced which are not circular would make quantitation very difficult. Thus, a study of the zonal characteristics and the establishment of a calibration curve are essential when this method is tested on tissues from any animal species.

Conclusions

A sensitive and reliable method for monensin detection in samples of various poultry tissues was developed. All extraction, plating, and bioautographic conditions were optimized for monensin. Silanization of experimental glassware prior to extraction, the correct solvent mixtures for extraction and plate development, as well as a variety of other conditions were found to play important roles in increasing the amount of monensin recoverable and detectable. Consistent results were a reflection of using standardized materials and calibrated equipment.

In summary, this method proved to be sensitive (detection limit 250 μg/kg) and reliable (99%) for monensin detection in poultry tissue samples (cardiac muscle, skeletal muscle, liver, kidney). It also proved to be fast and simple in terms of applicability when compared to other TLC/B procedures presently available. A minimum amount of labor and solvents are also required when compared to other TLC/B methods. Thus, this procedure is well suited for the quantitative detection of monensin in poultry tissues when analysis of a large number of samples is required. This method is also useful for the extraction, separation and detection, using a modified developing solvent system, of the ionophores lasalocid and salinomycin.

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Multiresidue Method for Isolation and Liquid Chromatographic Determination of Seven Benzimidazole Anthelmintics in Milk

AUSTIN R. LONG, LILY C. HSIEH, MARSHA S. MALBROUGH, CHARLES R. SHORT, and STEVEN A. BARKER[1]

Louisiana State University, School of Veterinary Medicine, Department of Veterinary Physiology, Pharmacology and Toxicology, Baton Rouge, LA 70803

A multiresidue method for the isolation and liquid chromatographic determination of 7 benzimidazole anthelmintics (thiabendazole, oxfendazole, para-hydroxyfenbendazole, fenbendazole sulfone, mebendazole, albendazole, and fenbendazole) in milk is presented. Blank or benzimidazole-spiked milk samples (0.5 mL) were blended with octadecylsilyl (C-18, 18% load, end-capped) derivatized silica packing material. A column made from the C-18/milk matrix was first washed with hexane (8 mL), and then the benzimidazoles were eluted with methylene chloride–ethyl acetate (1 + 2, v/v; 8 mL). The eluate contained benzimidazole analytes which were free from interfering compounds as determined by UV detection (photodiode array, 290 nm). Correlation coefficients of standard curves for individual benzimidazoles isolated from spiked samples were linear (0.989 ± 0.003 to 0.998 ± 0.001) with recoveries ranging from 70 ± 9% to 107 ± 2% for the concentration range (62.5–2000 ng/mL) examined. The inter-assay variabilities ranged from 4 ± 1% to 9 ± 7% with intra-assay variabilities of 3–6%.

The benzimidazole anthelmintics are used in animal production to control internal worm parasites and have become an integral part of the animal producing industry. For this reason, tissue residue monitoring programs have been established for many of these compounds to ensure that animal-derived human foods do not contain residues that exceed legal maximal levels (800 ng/g tissue) (1–3). An additional concern is the illegal use of some of these agents. For example, benzimidazole use in dairy cattle of breeding age is prohibited because this drug and its metabolites may manifest themselves as residues in milk obtained from treated animals, posing a health threat to consumers. Therefore, the monitoring of milk for benzimidazole residues has also been implemented.

In this regard we have been developing new methods for benzimidazole determinations in animal matrixes (4–9). As a result of these early studies, we have developed a method for multiresidue/multidrug class extractions of organophosphates, benzimidazoles, and beta-lactams from meat tissues (10), which overcomes some of the complications associated with classical isolations. We report here a further examination of this methodology, which we have named matrix solid-phase dispersion (MSPD), for the isolation and liquid chromatographic (LC) determination of benzimidazoles in milk.

Experimental

Apparatus and Reagents

(a) *Solvents.*—(LC grade) highest purity available from commercial sources; used without further purification.

(b) *Water.*—For LC analyses; triple-distilled and passed through Modulab Polisher I (Continental Water Systems Corp., San Antonio, TX) water purification system.

(c) *Benzimidazoles.*—Oxfendazole, fenbendazole, fenbendazole sulfone, and para-hydroxyfenbendazole (Hoeschst Corp., Frankfurt, Germany); mebendazole (Janssen Pharmaceutical, Inc., Piscataway, NJ); thiabendazole (Merck Sharp and Dohme, Rahway, NJ); and albendazole (J. O'Rangers, Center for Veterinary Medicine, Food and Drug Administration, Rockville, MD).

(d) *Column material.*—Bulk C-18 (40 μm, 18% load, end-capped; Analytichem International, Harbor City, CA) was cleaned by making a column (50 mL syringe barrel) of the bulk C-18 material (22 g) and sequentially washing with 2 column volumes each of hexane, methylene chloride (DCM), and methanol. Washed C-18 was vacuum-aspirated until dry.

(e) *Stock benzimidazole solutions.*—1000 μg/mL prepared by dissolving standard compounds with LC grade dimethylformamide (DMF) and diluting to desired μg/mL levels with DMF.

(f) *Sample extraction columns.*—Ten mL syringe barrels, thoroughly washed and dried prior to use.

Extraction Procedure

Milk (3.2% homogenized, pasteurized) samples were obtained from a local market. Two g C-18 was placed in a glass mortar and an aliquot (0.5 mL) of milk was placed directly onto the C-18. Standard benzimidazoles (10 μL, 3.125–100 μg/mL) and internal standard mebendazole (10 μL, 12.5 μg/mL) were added to the milk and samples were allowed to stand 1 min. Blank milk samples were prepared similarly except that 20 μL DMF containing no benzimidazoles were added to the sample.

Samples were then gently blended into the C-18 with a glass pestle until mixture was homogenous in appearance. Gentle circular motion with very little pressure was required to obtain a homogenous mixture. The resultant C-18/milk matrix was placed into a 10 mL plastic syringe barrel that was plugged with a filter paper disc (Whatman No. 1). Column head was covered with filter paper disc and column contents were compressed to final volume of 4.5 mL with syringe plunger from which rubber end and pointed plastic portion had been removed. A pipet tip (100 μL) was placed on the column outlet to increase residence time of eluting solvents on the column.

Resulting column was first washed with 8 mL LC grade hexane. If flow through column was hindered, positive pressure was applied to column head (pipet bulb) to initiate gravity flow. When flow had ceased, excess hexane was removed from column with positive pressure as described above. Benzimidazoles were then eluted with 8 mL methylene chloride–ethyl acetate (1 + 2, v/v) as described for hexane. Extract was dried under steady stream of dry nitrogen gas, and 0.1 mL methanol and 0.4 mL 0.05N H_3PO_4 were added. Sample was sonicated (5–10 min) to disperse residue, which resulted in a suspension. This was transferred to a micro-centrifuge tube and centrifuged (Fisher Microcentrifuge Model 235, Fisher Scientific, Pittsburgh, PA) 5 min at 13 000 × *g*. The clear supernatant liquid was filtered through a 0.45 μm filter (Prep Disc, Bio Rad, Richmond, CA) and an aliquot (20 μL) was analyzed by liquid chromatography.

Liquid Chromatographic Analysis

Analyses of extracted sample and standard benzimidazoles were conducted utilizing Hewlett-Packard HP 1090 HPLC (HP 79994A HPLC Chemstation) equipped with

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[1] Address correspondence to this author.

Table 1. Results for multiresidue method for determination of 7 benzimidazoles[a]

Compound	Corr. coeff., %	Rec., %	Inter-assay var., %	Intra-assay var., %
Thiabendazole	0.995 ± 0.002	88.7 ± 5.8	7.5 ± 5.4	2.9
Oxfendazole	0.989 ± 0.003	107.3 ± 2.4	4.6 ± 1.8	2.6
p-Hydroxy-fenbendazole	0.998 ± 0.001	94.4 ± 5.1	8.7 ± 6.7	1.9
Fenbendazole sulfone	0.998 ± 0.001	100.2 ± 5.1	4.1 ± 4.1	5.7
Mebendazole	Int Std	101.3 ± 5.1	4.1 ± 1.3	3.7
Albendazole	0.998 ± 0.001	81.0 ± 6.8	10.1 ± 8.2	3.3
Fenbendazole	0.999 ± 0.001	69.7 ± 8.9	9.5 ± 7.5	2.9

[a] For each compound examined (concentrations of 62.5, 125, 250, 500, 1000, and 2000 ng/mL; mebendazole internal standard, 250 ng/mL, 20 μL injection volume), correlation coefficients (± standard deviation, 5 standard curves, n = 5), percentage recovery (n = 30, 5 samples at each concentration), and inter-assay (n = 30, 5 samples at each concentration) and intra-assay variabilities of the 7 benzimidazoles isolated from spiked milk samples.

photodiode array detector set at 290 nm with bandwidth of 20 nm and reference spectrum range of 200–350 nm. Solvent system was 0.05N H_3PO_4–acetonitrile (60 + 40, v/v) at isocratic flow rate of 1 mL/min. A reverse-phase octadecylsilyl (ODS) derivatized silica column (10 μm, 30 cm × 4 mm id, Micro Pak, Varian, Sunnyvale, CA) maintained at 45°C was used for all determinations.

Peak area ratio (PAR) curves of standards and samples were obtained by plotting integration areas of generated peaks as a ratio to area of internal standard (mebendazole). Comparison of spiked sample PARs to PARs of pure standards run under identical conditions gave percentage recoveries. Inter-assay variability was calculated as follows: Mean of PARs for 5 replicates of each concentration (62.5, 125, 250, 500, 1000, and 2000 ng/mL) was calculated. Standard deviation (SD) corresponding to each of these means was divided by its respective mean which resulted in coefficient of variation for each concentration. Mean of these coefficients of variation was calculated along with standard deviation. Mean of coefficient of variation times 100 was defined as inter-assay variability plus or minus SD. Intra-assay variability was determined by standard error of mean PARs of 5 replicates of same samples.

Results

Table 1 gives the compounds examined, concentrations analyzed (62.5–2000 ng/mL), standard curve correlation coefficients (± standard deviation), percentage recoveries, and inter- and intra-assay variabilities of the 7 benzimidazoles isolated from spiked milk samples. Representative chromatograms of extracted blank and spiked milk samples are shown in Figure 1.

Discussion

Sample preparation is a critical part of many residue analyses. To effectively monitor benzimidazoles and other chemical residues that may be present in the food supply, efficient residue isolation protocols are needed. The trend in residue monitoring programs is the development of multiresidue/multidrug class isolation methods that minimize time and expendable materials. Ideally, these sample preparation protocols should involve few steps and result in extracts containing the target residue(s) with high recoveries and minimal background interferences. For example, the classical isolation technique (3) for the benzimidazole albendazole includes sample homogenization in large volumes of extracting solvents, several pH adjustments, back washing, additional solvent partitioning steps, and evaporation of large volumes of extracting solvents. This and other such classical residue isolation techniques may result in emulsion formation, which can lead to less than ideal recoveries of targeted residues. In general, this classical approach to drug residue analysis is time, labor, and materials intensive and is usually designed to isolate only one compound at a time. However, the results presented here, utilizing the MSPD method developed in this laboratory, indicate that this approach may overcome some of the limitations of classical isolation techniques outlined above for the isolation of drug residues from milk.

The methylene chloride–ethyl acetate (1 + 2, v/v) extract contained benzimidazole analytes that were free from interfering compounds when analyzed by liquid chromatography with UV detection (photodiode array, 290 nm) as can be seen in chromatograms of blank (Figure 1) and benzimidazole-spiked (Figure 1) milk samples. Standard curves for the benzimidazoles isolated from spiked milk samples were linear over the concentration range examined with average recovery percentages from 69.7% for fenbendazole to 107.3% for oxfendazole. The lowest percentage recovery for thiabendazole (83.2% at 62.5 ng/mL), oxfendazole (103.4% at 2000 ng/mL), para-hydroxyfenbendazole (87.5% at 125 ng/mL), fenbendazole sulfone (87.4% at 62.5 ng/mL), albendazole (72.2% at 250 ng/mL), and fenbendazole (61.2% at 500 ng/mL) was reasonable, especially when considering the fact that this is a multiresidue extraction method. The largest individual inter-assay variability for thiabendazole (20.3% at 250 ng/mL), oxfendazole (10.0% at 125 ng/mL), para-hydroxyfenbendazole (17.9% at 62.5 ng/mL), albendazole (25.5% at 62.5 ng/mL), and fenbendazole (24.4% at 62.5 ng/mL) demonstrate greater variability at the lower concentrations. However, the overall variability was acceptable and is reflected in the average (n = 30) inter-assay variabilities in

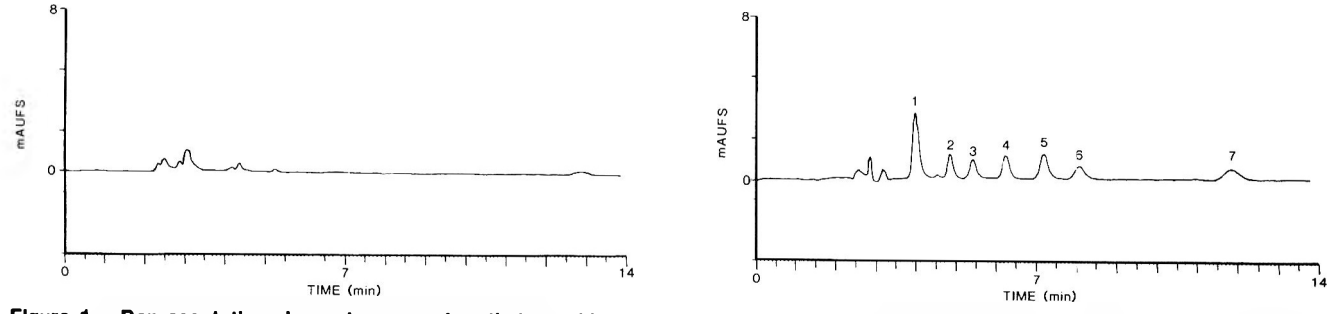

Figure 1. Representative chromatograms of methylene chloride–ethyl acetate (1 + 2, v/v) extract of blank (left); and benzimidazole-spiked (250 ng/mL, 20 μL injection volume) (right) milk samples. Order of elution is thiabendazole (1), oxfendazole (2), para-hydroxyfenbendazole (3), fenbendazole sulfone (4), mebendazole (5), albendazole (6), and fenbendazole (7).

Table 1. The intra-assay variabilities in Table 1 are representative of an identical sample (250 ng/mL, 20 μL injection volume) injected 5 times and thus reflect the variability associated with the analytical instrumentation.

The blending of the milk with C-18 disperses the sample over a large surface area and exposes the entire sample to the extraction process. Even though the volume of extracting solvents is only 8 mL, the process may be envisioned as an exhaustive extraction process whereby a large volume of solvent is passed over an extremely thin layer of sample. We theorize that the dispersion of the sample onto the C-18 packing material involves mechanical and hydrophobic forces. The nonpolar materials such as membrane lipids associate with the nonpolar C-18 polymer, facilitating the disruption and unfolding of the structural components of the milk matrix. The hydrophilic components and more polar protein ends would preferentially orient themselves away from the nonpolar lipid/C-18 inner regions and extend outward. Water and more polar constituents would associate with these hydrophilic ends. The theoretical aspects of this phenomenon have been presented (10) and scanning electron micrographs of uncoated and tissue coated C-18 beads support this hypothesis (unpublished observations).

A sequential elution protocol allows for the selective removal of potentially interfering compounds as well as the selective elution of target residues. The hexane wash removes lipid materials and neutral chromophores, which may interfere with subsequent benzimidazole analysis. A methylene chloride–ethyl acetate (1 + 2, v/v) solvent system was found to be most efficient in eluting the range of benzimidazoles examined. The 7 benzimidazoles could be eluted with methylene chloride but it was inefficient in eluting the entire range of benzimidazoles studied with acceptable recoveries. Ethyl acetate eluted all 7 benzimidazoles, but resulted in only a slight increase in recoveries. An elution sequence whereby the column was sequentially eluted with 8 mL each of methylene chloride and ethyl acetate, with the eluates combined, resulted in less than ideal recoveries of the internal standard mebendazole and thiabendazole. A 1:1 ratio of methylene chloride to ethyl acetate increased the recovery of all the benzimidazoles except thiabendazole.

Results presented here are based on spiked samples, much as would be required and obtained for the preparation of standard curves or for conducting recovery studies for the quantitative analysis of drug residues in milk incurred from the administration of a drug. The purpose of the present study was to examine the application of matrix solid phase dispersion for the isolation of the 7 benzimidazoles from a single milk sample, demonstrating the prospect that such methodology may be used to screen for a wide range of drugs in a single sample. While an examination of milk from animals actually administered these 7 benzimidazoles would be ideal, such samples were not available to us and such a study is outside the scope and limits of practicality of the present research. Studies examining incurred residues of individual benzimidazoles in milk by MSPD are currently being conducted under the auspices of the U.S. Food and Drug Administration.

The MSPD method may be suitable for benzimidazole isolations from other tissues or matrixes. The 62.5 ng/mL levels (20 μL injection volume, 1.25 ng on-column) determined are lower than the maximal legal permissible level of fenbendazole (800 ng/g) and thiabendazole (100 ng/g) in liver. Because of the cleanliness of the extract, sensitivity may be increased by increasing injection volume and/or dissolving the extract residue in a smaller final volume. Isolations of other drug residues from milk may be enhanced by the use of this method and are currently being conducted.

The MSPD method eliminates many of the problems associated with classical isolation techniques. It uses small sample sizes, has a minimal number of steps and no chemical manipulations in terms of pH adjustments, and requires a minimal amount of solvent. The savings in terms of time and solvent requirements make this procedure an attractive alternative to classical isolation methods.

Acknowledgments

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DRUGS IN FEEDS

Modified Liquid Chromatographic Method for Determination of Gentian Violet in Animal Feed

ELIZABETH E. MARTINEZ and WILBERT SHIMODA
Food and Drug Administration, Animal Drug Research Center, Denver Federal Center, Denver, CO 80225-0087

A modified liquid chromatographic method is described for the determination of Gentian Violet (GV) in animal feed. The reliable detection limit is 0.5 ng (reference standards), and 1 ppm GV was reliably determined in feed. The calibration curve was linear between 1 and 40 μg/mL. The method, developed in a study by the National Center for Toxicological Research, was modified to use methanol–water (9 + 1) instead of benzene–methanol as the eluting solution in the column cleanup. GV is extracted from feed with methanol–1N HCl (99 + 1), cleaned up on a Sephadex LH-20 column to remove any remaining interferences, separated on a Nova-Pak C_{18} column fitted with a precolumn filter, and determined at 588 nm. The identity of GV is confirmed by thin-layer chromatography (R_f = 0.47) by comparison with a reference standard. Average recoveries from 3 sets of 5 feed samples containing 2.5, 5.0, and 10.0 ppm GV were 115, 95, and 102%, respectively.

Gentian Violet (GV) (Figure 1), also known as Crystal Violet, is classified as a triphenylmethane dye. Some triphenylmethane dyes have been recognized as animal carcinogens. GV has been used to control fungi and intestinal parasites in humans, as an antimicrobial agent in treatment of burn victims, and as a vaginal cream and a pH indicator. At present, the U.S. Food and Drug Administration (FDA) permits the use of GV (8 ppm) in poultry feeds as a mold inhibitor.

A Dialog 2 literature search revealed that GV has been used to develop methods for the determination of thallium in gold (1), sulfite in sugar (2), boron in plants and water (3), and to detect some organic compounds (4). GV has been separated and quantitated by liquid chromatography (LC) (5) and separated and identified by thin-layer and column chromatography (6); it has been determined potentiometrically (7) and spectrophotometrically (2) and by mass spectrometry (8).

A study was conducted by the National Center for Toxicological Research (NCTR), Jefferson, AR, to investigate potential human hazards associated with the use of GV in food-producing animals. The study involved methods to estimate quantities of GV in human urine, wastewater, and animal feed (9). An LC method was developed by NCTR to ensure that GV added to the animal feed samples was uniformly distributed and stable during the preparation and feeding period of the study. The LC method was satisfactory but labor intensive; the estimated levels of GV in the feed samples ranged from 0.010 to 1000 ppm (9).

Because no other method was available, we modified the NCTR method in order to quantitate GV in chick feed. Methanol–water is used rather than benzene–methanol in the column cleanup step to avoid use of benzene, a known carcinogen. GV identity is confirmed by thin-layer chromatography (personal communication, J.E. Roybal, FDA, Animal Drug Research Center, Denver, CO).

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METHOD

Reagents

(a) *Methanol.*—Distilled-in-glass (Burdick & Jackson Laboratories, Inc., Muskegon, MI 49442).

(b) *Potassium phosphate, monobasic.*—Mallinckrodt, Inc., Paris, KY 40361.

(c) *Water.*—Distilled and deionized, filtered through 0.45 μm membrane filter (Millipore Corp., Bedford, MA 01730).

(d) *Sephadex LH-20.*—25–100 μm (Pharmacia Fine Chemicals, Piscataway, NJ 08854). Use 5 g dry powder per column.

(e) *LC mobile phase.*—Methanol–buffer (85 + 15).

(f) *Buffer.*—0.01M monobasic potassium phosphate adjusted to pH 3.0 with phosphoric acid.

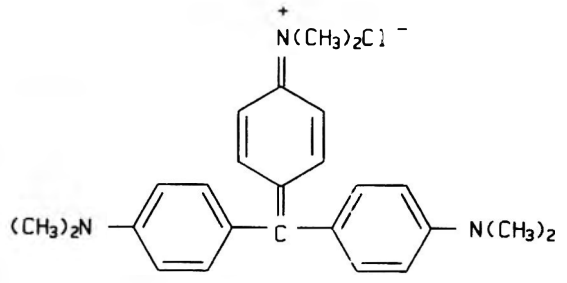
(g) *Sephadex column eluant.*—Methanol–water (9 + 1).

(h) *TLC mobile phase.*—Methanol–acetic acid (100 + 5).

(i) *GV reference standard.*—Crystal Violet, label declaration 93% purity (Lot 106F-3508, Sigma Biochemical, PO Box 14508, St. Louis, MO 63178), obtained from the Center for Veterinary Medicine (CVM). *Note:* Label declaration of 93% reflects concentration data obtained by CVM from supplier. If reference standard is declared to be less than 100% pure, weigh enough reference standard to make stock solution equivalent to 1000 μg/mL in 10 mL. Use reference standard to prepare following standard solutions. (*1*) *Stock solution.*—1000 μg/mL. Weigh sufficient GV into 10 mL volumetric flask and dilute to volume with anhydrous methanol. Store in dark, cool place. Discard after 5 days. (*2*) *Intermediate solution.*—10.0 μg/mL. Pipet 1 mL stock solution (*1*) into 100 mL volumetric flask and dilute to volume with anhydrous methanol. (*3*) *Working solutions.*—2.5 and 5.0 μg/mL. Pipet 2.5 and 5 mL intermediate solution (*2*) into separate 10 mL volumetric flasks, and dilute to volume with anhydrous methanol. Prepare fresh before each use.

Apparatus

(a) *Chromatographic tube.*—300 × 19 mm id with 250 mL reservoir (No. K-420280, Kontes Co., Vineland, NJ 08360).



hexamethylpararosaniline chloride

Gentian Violet C. I. 42555

Figure 1. Structure of Gentian Violet.

(b) *Receiving flask.*—50 mL pear-shape (No. K-608700, Kontes Co.).

(c) *Centrifuge tube.*—15 mL, graduated (VCR Scientific, PO Box 39396, Denver, CO 80239).

(d) *TLC plates.*—20 × 20 mm K-6 Whatman silica gel (Cat. No. 4860-820, Whatman Laboratory Products, Inc., Clifton, NJ 07014), or equivalent.

(e) *Vacuum rotary evaporator.*—Rotavapor R110 (Brinkmann Instruments, Inc., Westbury, NY 11590), water bath temperature 48–50°C.

(f) *Liquid chromatograph.*—Hewlett-Packard Model 1090 equipped with built-in diode-array detector, variable volume injection system, analytical workstation, and ThinkJet printer (Hewlett-Packard, Englewood, CO 80110), or equivalent instrumentation. Operating conditions: LC mobile phase at room temperature; flow rate 0.75 mL/min; injection volume 3.0 μL; sample wavelength 588 nm; reference wavelength 425 nm; threshold 10 μA; peak width 0.500 min; stop time 6.0 min; mobile phase, isocratic. Parameters for workstation monitor: window 10 min; range 10 mA/FS; sample interval 640 ns; spectrum range 210–400 nm; zero setting 10%.

(g) *LC column.*—Nova-Pak, 15 cm × 3.9 mm id stainless steel packed with 4 μm RP-C_{18} (Waters Chromatography Division, Millipore Corp., Milford, MA 01757).

(h) *Direct-connect guard column.*—Packed with pellicular reverse phase C_{18} packing material and attached to Nova-Pak column (Alltech Associates, 2051 Waukegan Rd, Deerfield, IL 60015).

(i) *Analytical evaporator.*—N-Evap (Organomation Associates, Inc., Northborough, MA 01532), water bath temperature 48–50°C, with nitrogen flowing into each tube.

(j) *TLC developing tank.*—Glass, lined with filter paper sheets.

(k) *Sephadex LH-20 column.*—Prepare as follows: Weigh 5.0 g Sephadex LH-20 into 150 mL beaker. Add 100 mL methanol–water (9 + 1). Let equilibrate 4 h and then pour contents into 300 × 19 mm id chromatographic tube. Do not restrict flow. Wash beaker with additional methanol–water (9 + 1) to ensure complete transfer of Sephadex LH-20 and to make surface on top of column bed uniform. Retain ca 3 cm eluant above column bed. After each use, wash column with 100 mL anhydrous methanol. Regenerate column with 100 mL methanol–water (9 + 1), retaining ca 3 cm eluant above column bed. Let column equilibrate 1 h before reusing.

Samples

Grind feed to pass 1 mm sieve and mix at least 24 h by using revolving mixer. (This step is required for finished feed, control feed, and spiked feed.)

Process blank feed control and feed spiked with 5.0 μg/g with each set of samples analyzed.

Extraction

Weigh 10 g finished feed into 250 mL glass centrifuge bottle with ground-glass stopper. Add 50 mL methanol–1N HCl (99 + 1) and stopper tightly. Shake mixture vigorously 20 min on mechanical shaker. Centrifuge 15 min at 2000 rpm. Decant extract into 150 mL beaker and save. Repeat extraction with additional 50 mL portion of methanol–1N HCl (99 + 1). Combine 50 mL extracts, and mix well. Transfer aliquot of extract equivalent to 5.0 μg GV to 50 mL pear-shape flask and evaporate to dryness by using rotary evaporator with 48–50°C water bath.

Sephadex LH-20 Column Chromatography

Transfer residue to Sephadex LH-20 column with 2 mL portion of methanol–water (9 + 1). Wash flask with three 2 mL portions of methanol–water (9 + 1), and add washings to column. Let each 2 mL portion enter column bed. Then add additional 6 mL methanol–water (9 + 1) to flask as final wash, and transfer washings to column. Let solution pass through column, and discard eluate. (Volume of eluate is ca 14 mL, and purple color is ca 2 cm from bottom of column at this point in procedure.) Add 6.0–6.5 mL methanol–water (9 + 1) to column, and collect eluate in 15 mL graduated centrifuge tube. Wash tip of column with ca 1.0 mL methanol–water (9 + 1), add washings to centrifuge tube, and vortex mix. Evaporate contents of tube to ca 0.5 mL by using nitrogen gas and 48–50°C water bath. Add 2 mL ethanol to tube, and evaporate to dryness by using nitrogen gas and 48–50°C water bath. Repeat process of adding 2 mL ethanol and evaporating until all water is removed from extract. Pipet 1.0 mL anhydrous methanol into centrifuge tube, and vortex mix.

Determination

Transfer total volume to 1.0 mL LC vial and secure with metal cap. Inject 3.0 μL aliquot into liquid chromatograph, and obtain response as peak area percent or concentration in μg/mL. Use external standard equivalent to 5.0 μg GV/mL to calibrate instrument. Determine concentration of GV in sample by comparing response obtained for sample with that obtained for reference standard.

TLC Confirmation

Score TLC plate 3 cm from top. Spot 10 μL each of reference standard and feed extract equivalent to 10 μg GV/mL ca 3 cm from bottom of plate. Place plate in developing tank that contains 100 mL methanol and 5 mL acetic acid. Let developing solvent migrate to top of scored plate. Re-

Table 1. Recovery of Gentian Violet from chick feed that contains animal fat[a]

Detn	Added, ppm	Found, ppm	Rec., %
1	2.5	3.0	119
2	2.5	2.8	111
3	2.5	2.8	110
4	2.5	2.9	115
5	2.5	3.0	122
Av. rec., %			115
CV, %			4.1
6	5.0	4.9	98
7	5.0	4.5	91
8	5.0	4.7	95
9	5.0	4.5	91
10	5.0	4.9	98
Av. rec., %			95
CV, %			3.8
11	10.0	9.8	98
12	10.0	10.0	100
13	10.0	10.0	100
14	10.0	10.4	104
15	10.0	10.7	107
Av. rec., %			102
CV, %			3.5

[a] 10 g sample size.

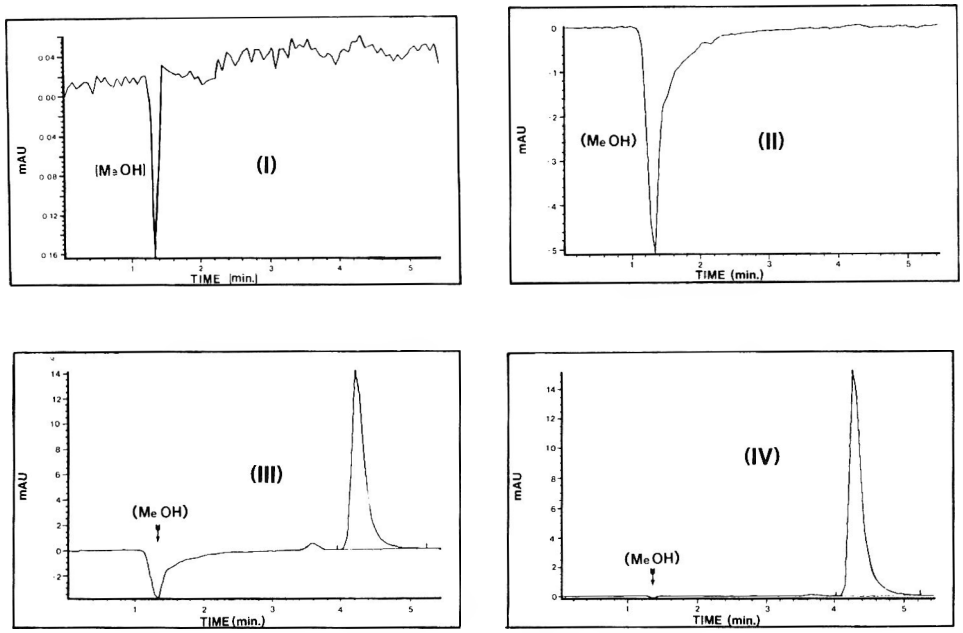


Figure 2. Liquid chromatograms of (I) reagent control, (II) feed control blank, (III) feed spiked at 5.0 μg/g with Gentian Violet (GV) and (IV) reference standard equivalent to 5.0 μg GV/mL. Three μL aliquot injected into liquid chromatograph. Retention time of GV in spiked feed = 4.23 min; retention time of GV reference standard = 4.16 min.

move TLC plate from developing tank and air-dry 3–4 min. Measure height of purple spots (GV) to obtain R_f value, which is ca 0.47 for GV. For further confirmation, obtain UV scans by using Hewlett-Packard Model 1090 liquid chromatograph, or equivalent instrument.

Results and Discussion

The modified method described is fast and uses methanol rather than benzene, a known carcinogen, in the column cleanup procedure.

Fortified chick feed that contained animal fat was supplied by CVM, Beltsville, MD, and used to obtain recovery data; 10 g feed samples spiked with 2.5, 5.0, and 10.0 ppm GV were analyzed in sets of 5. The recovery data are shown in Table 1. The average recoveries from feed spiked with 2.5, 5.0, and 10.0 ppm GV were 115, 95, and 102%, respectively; the corresponding coefficients of variation were 4.1, 3.8, and 3.5%. These results were calculated from separate analyses of 5 simultaneously prepared samples of each concentration. The liquid chromatograph was calibrated by using GV reference standards equivalent to each concentration of spiked feed, i.e., 2.5, 5.0, and 10.0 μg/mL. The results were obtained as peak area percent or concentration in μg/mL. Spiked feed extracts were also analyzed by using a liquid chromatograph with a variable wavelength detection system; the results were comparable to those obtained with the liquid chromatograph with a fixed wavelength.

The initial analyses during method development gave lower recoveries and demonstrated poor precision. These results were traced to 3 possible factors, and the appropriate changes were made in the method. First, the feed must be ground to pass a 1 mm sieve instead of the 2 mm sieve that was originally used, and the feed must be mixed overnight (24 h) to obtain homogeneity. Second, the feed should not be mixed in a plastic bag because this procedure tends to produce a static charge that causes GV to adhere to the bag. Third, two 50 mL extractions give better results than one 100 mL extraction.

When the above refinements were used, good recoveries and reasonable coefficients of variation were obtained. A feed spiked at 1.0 μg/g gave a recovery of 116% with a coefficient of variation of 9.2%. (GV is visible on the Sephadex LH-20 column at this concentration.) For concentrations >10 ppm, 1–3 mL more eluting solution is required to recover all visible GV from the Sephadex LH-20 column.

Because this LC method is fast, 10 feed samples can be analyzed in 1 day. The detection limit is 3 ng (reference standards) and 10 ng in feed. The calibration curve is linear between 1 and 40 μg/mL. Different types of liquid chromatographs were found to give similar results. Our data were obtained from analyses of spiked feed extracts with 3 μL aliquots injected into the liquid chromatograph. GV was well separated from feed interferences on the reverse-phase C_{18} Nova-Pak column.

Figure 2 shows chromatograms of a reagent control, a feed control blank, a feed spiked with 5.0 μg GV/g, and a reference standard equivalent to 5.0 μg GV/mL. The solvent front appears as a negative peak because of the 425 nm reference wavelength. GV identity was confirmed by the thin-layer chromatography procedure and by UV scans obtained with the Hewlett-Packard Model 1090 and Model 1084B liquid chromatographs.

In summary, this LC method, which is fast, gives good recoveries and acceptable coefficients of variation, and uses common laboratory equipment and solvents, gave similar results when tested by 2 different analysts.

Acknowledgments

We are grateful to Jeffrey A. Hurlbut for his untiring technical assistance and guidance; to Jose E. Roybal for permission to use the TLC procedure; to Alice L. Marcotte for editorial assistance; and to CVM for the animal feeds and GV reference standard. We also thank Dorothy H. Hughley for typing the manuscript.

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EGGS AND EGG PRODUCTS

Gas Chromatographic Determination of Cholesterol in Egg Products

J. D. BEYER, F. X. MILANI, M. J. DUTELLE, and R. L. BRADLEY, JR
University of Wisconsin, Department of Food Science, Madison, WI 53706

A method has been developed for quantification of cholesterol in fresh egg yolks, spray-dried egg yolks, fresh whole eggs, and spray-dried whole eggs. The method uses saponification followed by petroleum ether extraction of cholesterol. Separation of organic and aqueous layers is enhanced by sodium chloride. Petroleum ether extracts are dried under nitrogen and redissolved in chloroform–methanol (2 + 1) for injection into a gas chromatograph. Cholesterol is separated and quantitated on a high temperature capillary column coated with 5% diphenyl and 95% dimethyl polysilicone crosslinked gum. The method was compared with the current AOAC method 17.017–17.022, and results indicated no significant difference (α = 0.05). However, the proposed method allowed separation and analysis of 16 samples in 7 h while the current AOAC method allowed separation and analysis of only 4 samples in 9 h.

Cholesterol, a steroid alcohol, is a precursor of bile acids, steroid hormones, and provitamin D_3 in humans. The public has been advised by the Surgeon General to reduce its cholesterol intake (1). Target foods are meats, dairy products, and eggs. Methods to analyze these products are necessary. The current AOAC method for determination of total cholesterol in egg yolks, **17.017–17.022** (2) is labor intensive and can not be easily automated. Thus, an equivalent quantitative method that would extract cholesterol from eggs, but would require less glassware, reagents, and time, is desirable.

Cholesterol comprises approximately 99% of the total steroid content in egg yolks (3). Several methods have been used to quantitate total cholesterol content in multicomponent foods (4–6), blood (7–9), and eggs (10–12). Methods usually involve an organic extraction and a quantitative technique such as gravimetry, titrimetry, colorimetry, thin-layer chromatography, enzyme assay, liquid chromatography, radioassay, or gas chromatography (for an extensive review, see Sweeney and Weihrauch (13)). Gas chromatography (GC) and liquid chromatography (LC) apparently are more precise than are other quantitative techniques. GC systems have a faster recycle time than do LC systems, and GC analysis is particularly rapid when an automatic sampling device is used.

A method for plasma cholesterol determination by Abell et al. (7) has been used to quantitate total cholesterol content in egg yolks (10). This method requires alcoholic potassium hydroxide saponification followed by petroleum ether extraction and quantitation by colorimetry. The method is rapid and simple compared with AOAC method **17.017–17.022** (2) for determining total cholesterol content in fresh egg yolks. However, when the method was used for dried egg yolks, the sample remained lumpy after saponification and a 3 phase separation consistently developed, which prevented quantitative removal of the petroleum ether phase.

A method was developed using alcoholic potassium hydroxide saponification (6), petroleum ether extraction of cholesterol, and quantitation by gas chromatography (4). Saponification and petroleum ether extraction were also proposed by Sperry and Brand (14) for determination of cholesterol in plasma. Saponification and petroleum ether extraction steps were designed to handle the quantities of triglyceride and lipoprotein encountered in extracting total cholesterol in dried egg yolk products.

The proposed method uses a more concentrated alcoholic potassium hydroxide solution and a higher temperature than that of the previously described method, but a shorter time interval than the AOAC method. The proposed method uses sodium chloride to yield a 2 phase separation of petroleum ether and aqueous layers. Also, this method uses petroleum ether, while the AOAC method uses diethyl ether. The Punwar method (4) uses chloroform–methanol extracts and a separatory funnel to collect the saponifiable aqueous layer. In the present study, the proposed method developed for quantitative extraction of total cholesterol in eggs was compared with the AOAC method.

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METHOD

Apparatus

(a) *Chromatograph.*—Hewlett-Packard Model 5890 gas chromatograph equipped flame ionization detector and autosampler. Operating conditions: 1:40 split ratio; helium carrier gas flow 0.75 mL/min; 1 μL injection; injector 325°C; detector 325°C; oven 275°C.

(b) *Integrator.*—Hewlett-Packard Model 3393A.

(c) *Column.*—15 m × 0.25 mm id DB 5 (J & W Scientific, Folsom, CA) capillary column. 5% diphenyl and 95% dimethyl polysilicone crosslinked gum, film thickness 0.1 μm. Solvent peak appeared at 0.8 min; cholestane 1.9 min; cholesterol 2.8 min.

Reagents

(a) *Concentrated potassium hydroxide solution (8.56M).*—Dissolve 60 g KOH in 40 mL water.

(b) *Ethanolic potassium hydroxide solution (2.14M).*—Mix 25 mL concentrated KOH solution and 75 mL 95% ethanol. Make new solution daily and use only top 90 mL of prepared solution.

(c) *Saturated sodium chloride.*—Dissolve excess NaCl in water, heat gently, and let solution stand overnight.

(d) *Internal standard solution.*—Dissolve 10 mg 5 alpha-cholestane/mL in 2,2,4-trimethylpentane, final concentration 1 mg/mL.

(e) *Petroleum ether.*—Boiling point 35–60°C; ACS grade (Mallinckrodt, Inc., Science Products Div., St. Louis, MO 63134).

Procedure

Accurately weigh (± 0.001 g) 0.5 g liquid egg yolk or 0.25 g dry egg yolk into 50 mL glass centrifuge tube with rubber-lined screw cap (Fisher Scientific Co., West Haven, CT 06516). Add 10 mL alcoholic KOH solution, cap tightly, and vortex-mix 20 s. Hold 1 h in 70 ± 2°C water bath. Agitate well every 15 min. Cool to room temperature. Add 10 mL petroleum ether, 5 mL saturated NaCl solution, 1 mL internal standard solution and 5 mL water, and vortex-mix 20 s. Centrifuge (1571*g*) 10 min or until emulsion breaks and 2 clear layers develop. Remove upper layer with Pasteur pipet

Table 1. Comparison of AOAC method with the proposed method for determining cholesterol in fresh egg yolk, spray-dried egg yolk, fresh whole egg, and spray-dried whole egg

Statistic[a]	AOAC method	Proposed method
Fresh egg yolk:		
Av., mg/mL	12.4	12.3
n	12	12
SD	0.286	0.406
CV, %	2.32	3.31
	NS[b]	
Spray-dried egg yolk:		
Av., mg/mL	21.5	21.2
n	12	12
SD	0.918	0.932
CV, %	4.27	4.40
	NS	
Fresh whole egg:		
Av., mg/mL	4.22	4.16
n	12	12
SD	0.174	0.202
CV, %	4.13	4.85
	NS	
Spray-dried whole egg:		
Av., mg/mL	16.1	16.1
n	12	11
SD	0.622	0.652
CV, %	3.86	4.05
	NS	

[a] AV. = average of 12 replicates; n = number of replicates; SD = standard deviation; CV = coefficient of variation.
[b] Not significantly different at $\alpha = 0.05$ level.

and transfer to second tube. Re-extract twice with 10 mL portions of petroleum ether, centrifuge, and remove upper layer with Pasteur pipet. Evaporate 3 combined extracts under nitrogen in 50 ± 1°C water bath to about 1 mL, but not to dryness. Add 2 mL chloroform–methanol (2 + 1), and mix well. Quantitatively transfer concentrated cholesterol extract with Pasteur pipet to 10 mL volumetric flask. Rinse drying tube 3 times with 2 mL portions of chloroform–methanol (2 + 1). Dilute to volume with chloroform–methanol (2 + 1 v/v).

Chromatography

Internal standard was added for quantitation. Five standards from 0.25 to 2.0 mg/mL were used to determine linear relationship. Calculate cholesterol as follows:

$$\text{Cholesterol, mg/g sample} = (PA/PA') \times (W'/W) \times RF$$

where PA and PA′ = peak area of cholesterol and cholestane, respectively; W = weight of sample, g; W′ = weight of cholestane = 1 mg; and RF (response factor) = 1.12.

Experimental Design

Fresh egg yolks, spray-dried egg yolks, fresh whole eggs, and spray-dried whole eggs were analyzed in 12 replicates by each method.

Results and Discussion

After saponification, saturated salt solution was added to the sample to eliminate gel formation that developed during centrifugation of the dried egg products. Two clear layers with a distinct interface formed from centrifugation.

The results in Table 1 indicate a high degree of precision for both methods as shown by low coefficients of variation. The use of an autosampler in the GC system can be credited with the good repeatability obtained. Previous determinations of cholesterol in foods by Punwar (5) had coefficients of variation ranging from 8.5 to 23.2%. Coefficients of variation in this study varied from 2.32 to 4.27% for the AOAC method and 3.31 to 4.85% for the proposed method.

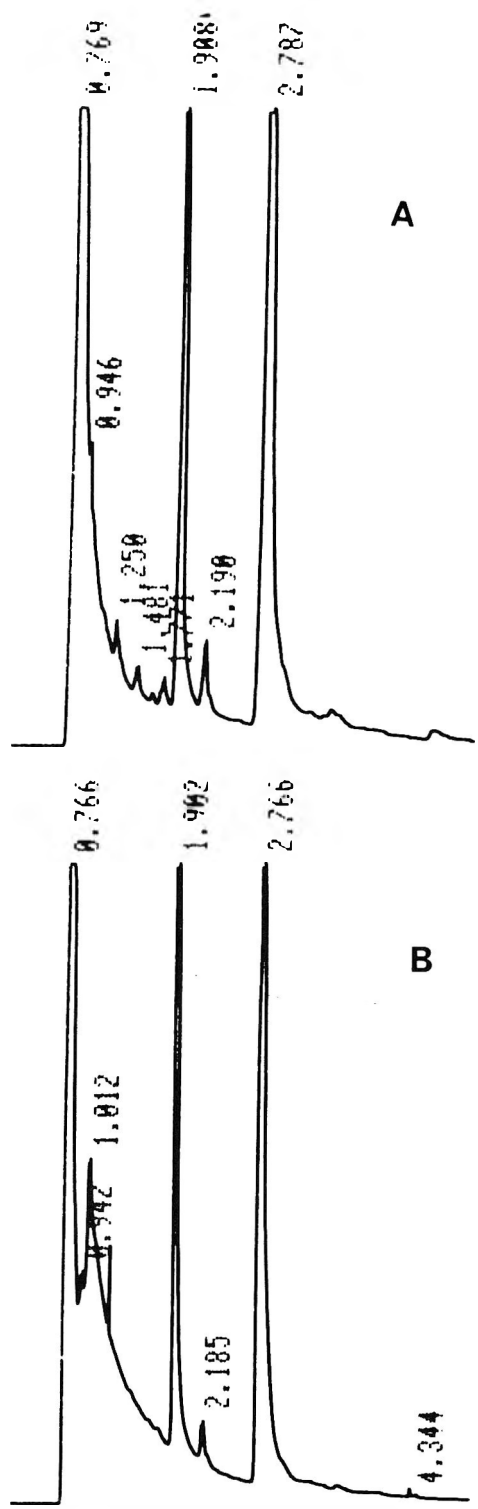

Figure 1. Gas chromatogram of AOAC (A) and proposed (B) methods for extracting cholesterol. Identification of peaks: 0.8 solvent, 1.9 cholestane, 2.8 cholesterol.

Differences in purity of the petroleum ether extract can be seen from the gas chromatograms in Figure 1. The gas chromatogram from the AOAC method had more peaks than that from the proposed method. The peaks are probably caused by small amounts of salts of free fatty acids.

A 2-sided Student *t*-test was used to determine statistical difference (15). At the 95% confidence level ($\alpha = 0.05$), no

significant difference was found between the 2 methods for the products analyzed.

The proposed method is more rapid; a technician can analyze 16 samples in 7 h; the AOAC method requires 9 h to analyze 4 samples.

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ENVIRONMENTAL ANALYSIS

Development and Validation of New Analytical Method for Acrolein in Air

AKIO YASUHARA,[1] K. JASON DENNIS, and TAKAYUKI SHIBAMOTO
University of California, Department of Environmental Toxicology, Davis, CA 95616

A new method was developed to determine vapor-phase acrolein in air samples. Air containing vapor-phase acrolein was purged into impingers filled with a dichloromethane solution of *N*-methylhydrazine. The resulting derivative, 1-methyl-2-pyrazoline, was analyzed by gas chromatography using a nitrogen-phosphorous detector (NPD). The detection limit was 8.9 pg 1-methyl-2-pyrazoline, equivalent to 5.9 pg acrolein. The recovery efficiencies of vapor-phase acrolein were 98.0 ± 2.9% and 100.3 ± 3.1% for 150 and 15 μg, respectively. This method was satisfactorily applied for determination of acrolein formed from various heated fats. The amounts of acrolein formed in a headspace were 109 μg/L from lard, 164 μg/L from corn oil, 5.1 μg/L from cotton seed oil, and 163 μg/L from sunflower oil.

Acrolein is a pungent liquid and produces intense irritation of the eye and mucous membranes of the respiratory tract. It has been reported in automobile exhaust (1) and in atmospheric air samples collected at heavy traffic areas (2–4). It is, therefore, suggested that a major source of acrolein in outdoor air is automobile exhaust (5). Acrolein has also been found in various foods (6). The formation of a large amount of acrolein heated cooking oil or beef fat was reported recently, suggesting that a major source of acrolein in indoor air is cooking (7).

The determination of acrolein is a difficult analytical procedure because the compound is highly volatile and polymerizes readily (8). Most analytical methods for acrolein involve derivatization with reagents such as 2,4-dinitrophenylhydrazine (9) or 4-hexylresorcinol (10) and subsequent quantitation by colorimetric techniques. These methods require tedious experimental steps and are not specific to acrolein. Recently, acrolein was determined as the 1-methyl-2-pyrazoline derivative with *N*-methylhydrazine in a study of the formation of malonaldehyde in photoirradiated corn oil (11). In the present study, the application and validation of the pyrazoline method for acrolein in air samples is investigated.

Experimental

Synthesis of Standard 1-Methyl-2-Pyrazoline

Suspend 18.5 g acrolein (Aldrich Chemical Co., Milwaukee, WI) in 40 mL dichloromethane (J.T. Baker Chemical Co., Phillipsburg, NJ) and then add 30 mL dichloromethane containing 13.8 g *N*-methylhydrazine (Aldrich Chemical Co., Milwaukee, WI). Stir reaction solution with magnetic stirrer for 1 h at 0°C. Separate organic layer in separatory funnel and dry over anhydrous sodium sulfate. Remove sodium sulfate and concentrate solution by 2 successive distillations at atmospheric pressure. Obtain 1-methyl-2-pyrazoline (ca 19.3 g) (purity = 97.6%) by fractional distillation at atmospheric pressure and 103–108°C. Spectral data for 1-methyl-2-pyrazoline are shown in Table 1. Use as standard to obtain GC calibration plot for acrolein analysis (12).

Examination of Linearity between Amount of Acrolein and GC Response

Dissolve 10 μL *N*-methylhydrazine in 10 mL dichloromethane and add various amounts of acrolein (150 ng–150 μg). Let reaction solution stand 1 h. Add 30 μL acetylacetone to reaction solution and let stand 30 min. Add 10 μL GC internal standard *N*-methylacetamide solution (12.2 mg/mL in dichloromethane) and inject 1 μL sample solution into GC apparatus. Calculate peak area ratio between 1-methyl-2-pyrazoline and internal standard. Typical results are shown in Figure 1.

Effect of Reaction Time on 1-Methyl-2-Pyrazoline Formation

Add 300 μg acrolein to 20 mL portions of dichloromethane solutions to which 20 μL *N*-methylhydrazine is added. Stop reaction after 5, 10, 20, 30, 40, 50, and 60 min by adding 50 μL acetylacetone to each reaction mixture. Stir solutions for 30 min before analysis.

Trapping Vapor-Phase Acrolein in Air

Fill each of 2 impingers connected in series with 20 mL dichloromethane; add 20 μL *N*-methylhydrazine to each. Place known amount of acrolein in air-tight plastic bag and let vapor equilibrate at room temperature. Purge an air sample containing vapor-phase acrolein into impingers with constant air stream (50 mL/min) at 0°C for 20 to 30 min. Add 50 μL acetylacetone to trapping solution. Adjust volume of the solution to 20 mL with dichloromethane and analyze trapping solution in each impinger for acrolein as 1-methyl-2-pyrazoline by GC with *N*-methylacetamide as internal standard.

When air stream (50 mL/min) was passed through 2 blank dichloromethane traps cooled at 0°C for 7 h, the amount of dichloromethane in the first and second traps decreased from 20 to 19.2 mL and from 20 to 19.5 mL, respectively. These results indicate that the amount of volatiles escaping from the traps during collection was negligible. However, the volume of the solution was always adjusted to 20 mL for quantitative analysis.

Determination of Acrolein from Heated Cooking Oils and Lard

Place 25 g cooking oil (Nisshin Foods Co., Tokyo, Japan) or lard (Taiyo Co., Yokohama, Japan) in flask. Heat flask to 180°C and purge headspace gas into impingers with air stream (50 mL/min) for 30 min. Determine acrolein as described above.

Instruments

A Hewlett-Packard Model 5890 A gas chromatograph (GC) equipped with an NP detector and a 30 m × 0.25 mm id bonded phase DBWAX fused silica capillary column (J&W Scientific, Folsom, CA) was used for quantitative analysis of 1-methyl-2-pyrazoline derived from acrolein and *N*-methylhydrazine. Oven temperature was held at 45°C for 2 min and then programmed to 170°C at 4°/min. Injector and detector temperatures were 250 and 300°C, respectively. Helium carrier gas flow rate was 44.6 cm/s. A Jeol Model JMS-DX 300

[1] Present address: National Institute for Environmental Studies, Yatabe-Machi, Tsukuba, Ibaraki, 305 Japan.

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Table 1. MS and NMR spectral data of 1-methyl-2-pyrazoline

MS: m/z (%)	84 (M^+, 75), 83 (100), 56 (31), 55 (10), 43 (34), 42 (80), 41 (18), 39 (15), 28 (57), 27 (12), 18 (12), 15 (23)
NMR	d 2.82 (3H, s, N—CH_3), d 6.78 (1H, t, N=CH—CH_2), d 2.66 (2H, m, CH—CH_2—CH_2), d 2.94 (2H, t, CH_2—CH_2—N), J (N=CH—CH_2) = 1.68 Hz, J (CH_2—CH_2—N) = 9.36 Hz

mass spectrometer and Jeol JNM-GX 400FT nuclear magnetic resonance spectrometer were used to obtain mass and NMR spectra of 1-methyl-2-pyrazoline, respectively.

Results and Discussion

Determination of detection limit of 1-methyl-2-pyrazoline by GC-NPD.—Figure 2 shows a GC tracing of 100 pg 1-methyl-2-pyrazoline. Generally, the detection limit is defined as the amount of analyte which produces a peak height more than 3 times the noise level. In this tracing, S (peak height of

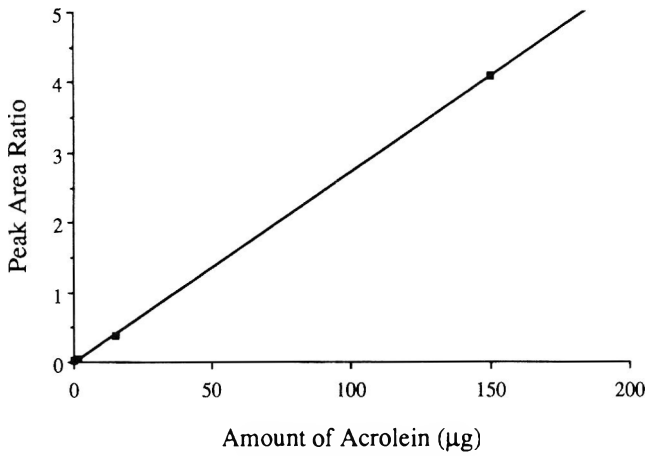

Figure 1. Linearity between amount of acrolein and GC response.

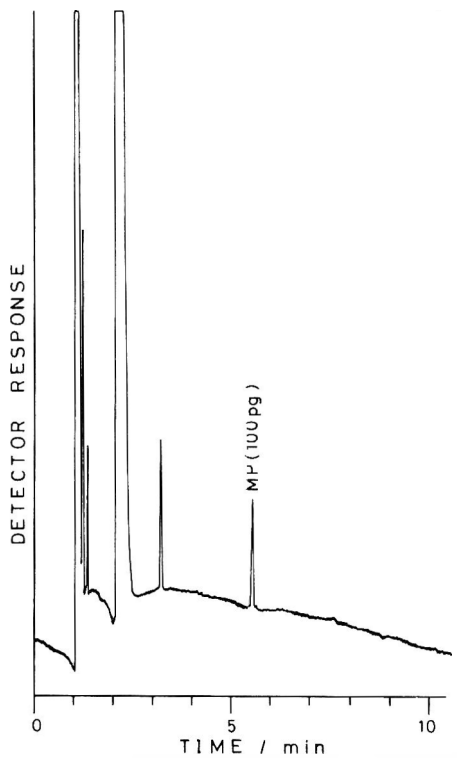

Figure 2. Gas chromatogram of 100 pg 1-methyl-2-pyrazoline (MP).

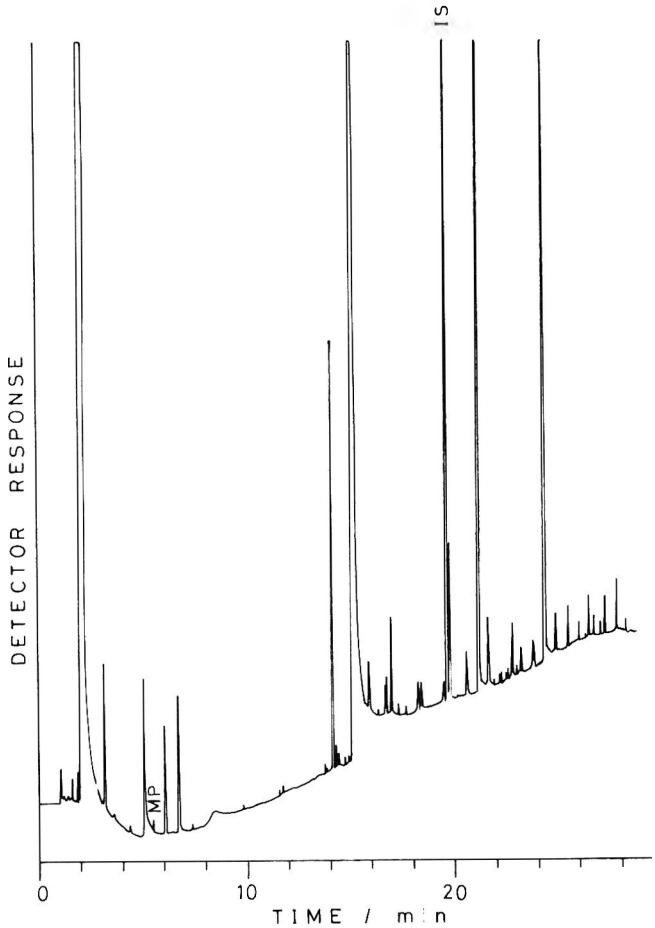

Figure 3. Gas chromatogram of dichloromethane solution of *N*-methylhydrazine (blank).

1-methyl-2-pyrazoline) = 27 mm; N (noise level) = 0.8 mm; and S/N (100 pg 1-methyl-2-pyrazoline) = 33.8. If S/N is 3 at lowest detection level, then lowest detection level of 1-methyl-2-pyrazoline is 8.9 pg (100 pg × 3/33.8), equivalent to 5.9 pg acrolein. However, if a blank value is larger than the noise level, the blank value should be used instead of N in the above equation.

Determination of GC blank value of reaction mixtures for 1-methyl-2-pyrazoline.—A GC tracing of a dichloromethane solution of *N*-methylhydrazine (Figure 3) shows a peak which has exactly same retention time as that of 1-methyl-2-pyrazoline. The signal caused by this chemical is equivalent to 284 ng of 1-methyl-2-pyrazoline in 10 mL trapping solution. If 284 ng of background 1-methyl-2-pyrazoline is present in 10 mL of the dichloromethane solution of *N*-methylhydrazine (1 μL/mL), then a 1 μL injection volume contains 28 pg. Since reliable values for precise quantitation by GC analysis require signals greater than 3 times the blank value, a 1 μL of sample should contain more than 84 pg 1-methyl-2-pyrazoline, equivalent to 56 pg acrolein in the present method.

The relationship between the amount of acrolein and GC

Table 2. Amount of acrolein found in headspace of 25 g heated fats

Fats	Concn in headspace, μg/L
Lard	109
Corn oil	164
Cotton seed oil	5.13
Sunflower oil	163

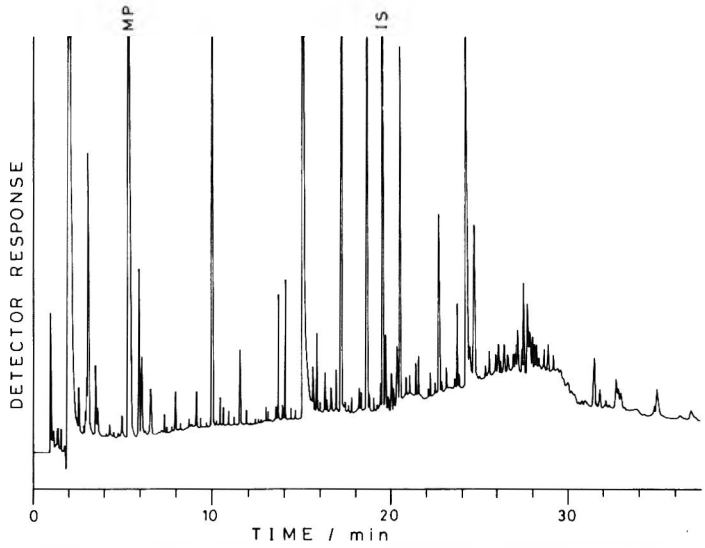

Figure 4. Gas chromatogram of headspace sample from heated lard.

response was linear for acrolein levels from 1.5 to 150 μg in 10 mL of a dichloromethane solution. However, the blank value becomes significant when the amount of acrolein is less than 150 ng in 10 mL (ca 5.64 ppb). If 1 μL of this solution is injected, 22 pg 1-methyl-2-pyrazoline is detected. This value is less than the NPD detection limit of 84 pg. Therefore, this method is not recommended for samples with 1-methyl-2-pyrazoline concentrations below 5.64 ppb. However, if the volume of dichloromethane solvent of a sample solution were reduced by distillation to increase the concentration of 1-methyl-2-pyrazoline to greater than 5.64 ppb, this method would still be applicable. Product yields over 90% were obtained in 5 min and the yield leveled off after 20 min. Therefore, 30 min reaction time is recommended.

Use of Acetylacetone.—Excess unreacted *N*-methylhydrazine decomposes into many products during GC analysis. It is, therefore, important to remove unreacted *N*-methylhydrazine immediately after the reaction with acrolein. Acetylacetone was used for this purpose because it reacts with *N*-methylhydrazine readily at room temperature. Also, the GC retention time of its derivative, trimethylpyrazole, is significantly different from that of *N*-methylpyrazoline. Over 95 and 98% of 20 μL *N*-methylhydrazine were reacted with 40 and 50 μL acetylacetone, respectively. The reaction efficiency remained constant when the amount of acetylacetone was increased to more than 60 μL. However, if the amount of acetylacetone exceeded 100 μL, unreacted acetylacetone overloaded the GC analysis. The optimum amount of acetylacetone to remove excess *N*-methylhydrazine in the trap was thus determined to be 50 μL.

Stability of N-methylhydrazine solution.—When a pure air stream (50 mL/min) was bubbled into 20 mL dichloromethane containing 20 μL *N*-methylhydrazine for 1 h, no significant changes in the *N*-methylhydrazine solution were observed, suggesting that bubbling air for prolonged period of time does not cause significant decomposition or oxidation of *N*-methylhydrazine.

Recovery efficiency of vapor-phase acrolein.—No 1-methyl-2-pyrazoline was detected in the second impinger in any experiments. Thus, all acrolein was trapped in the first impinger. Recovery efficiencies of vapor-phase acrolein were 98.0 ± 2.9% and 100.3 ± 3.1% for 150 and 15 μg, respectively. These values are averages of 4 replications ± standard deviation.

Analysis of acrolein formed from heated cooking oils and lard.—Table 2 shows the results of acrolein analysis in headspace samples from heated cooking oils and lard. Figure 4 shows a typical gas chromatogram of the headspace gas of heated lard trapped in the first impinger. Baseline separation of 1-methyl-2-pyrazoline (MP) was obtained. The method developed in the present study was proved to be useful for acrolein determination in air.

Acknowledgment

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Optimized Gel Permeation Chromatographic Cleanup for Soil, Sediment, Wastes, and Oily Waste Extracts for Determination of Semivolatile Organic Pollutants and PCBs

JEAN M. CZUCZWA[1]
Battelle Columbus Division, 505 King Ave, Columbus, OH 43201
ANN ALFORD-STEVENS
U.S. Environmental Protection Agency, Office of Research and Development, Environmental Monitoring Systems Laboratory, 26 Martin Luther King Dr, Cincinnati, OH 45268

A gel permeation chromatographic (GPC) method, used by the U.S. Environmental Protection Agency (USEPA), was modified for cleanup of soil, sediments, wastes, and oily wastes before determination of semivolatile organic pollutants. The modifications included new calibration procedures and control of the amount of material processed. The modifications were evaluated for soil and sediment matrixes in a 5-laboratory study where each laboratory processed a solution containing a phthalate, substituted phenols and benzenes, polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), nitroaniline, and pesticides. With the exception of nitroaniline, analyte recoveries were 87–112%, with relative standard deviations (RSDs) of 6.7–26%. Soil samples containing PCBs and fortified with 6 pesticides at 0.7–4 μg/g were also analyzed by the 5 laboratories. The mean recovery of the 6 pesticides was 100% with a mean RSD of 16%. Mean RSD for the determination of total PCBs was 8.9%. An additional modification for the processing of wastes and high concentration waste samples was attempted; this involved GPC processing of sample extracts dissolved in 1 + 1 butyl chloride–methylene chloride. This modification did not improve recoveries of the semivolatile analytes. Finally, the modified GPC protocol was applied to PCB-contaminated reclaimed waste oils samples. Two GPC cleanup steps were used to separate PCBs from the waste oil samples before PCBs were determined by gas chromatography combined with electron-capture detection (GC/ECD).

Gel permeation chromatography (GPC) is widely used to prepare extracts of environmental samples such as fish (1–3) and plant (4) tissue, and sludge (6), solid waste, soil, and sediment samples (6, 7). Presently, the U.S. Environmental Protection Agency (USEPA) uses GPC analysis in standard procedures (8–10) to prepare soil, sludge, and sediment extracts for determination of semivolatile organic pollutants by gas chromatography/mass spectrometry (GC/MS). The semivolatile analytes include phthalates; alkyl-, nitro-, and chlorophenols; polycyclic aromatic hydrocarbons (PAHs); polychlorinated biphenyls (PCBs); anilines; and pesticides and their metabolites. The GPC method was used to prepare many of the more than 20 000 organic samples analyzed in 1986 as part of the USEPA Contract Laboratory Program (CLP) of Superfund.

The USEPA GPC method was developed from an earlier method for the determination of pesticides in fish tissues (1, 2). In the fish tissue method, high molecular weight lipid material was separated from the lower molecular weight analytes with a 60 g Bio-Beads S-X3 (Bio-Rad) column and 1 + 1 cyclohexane–methylene chloride as the mobile phase. The present GPC method uses methylene chloride as the mobile phase to increase the solubility of the analytes.

Further characterization of the GPC method for application to soils, sediment, solid waste, and waste oil matrixes was desirable for a number of reasons. The change in mobile phase from cyclohexane/methylene chloride to 100% methylene chloride may affect calibration. The separation of high and low molecular weight soil components, such as elemental sulfur present in sediments, from pollutants may require different conditions than those needed for separation of high molecular weight lipids from pollutants. Irreversible adsorption of some soil components to the GPC column is observed as increasing discoloration of the column as it is used, but the extent of this adsorption is not known.

Column capacity is an important method parameter; up to 1 g fish lipid can be separated from analytes on the S-X3 column (1). However, GPC column capacity is related to retention volume, and the 1 g capacity for the early eluting lipid material may not be applicable to soil extracts containing nonlipid components. Finally, the effects of solvent and sample composition on column performance are not well understood.

The present report consists of 4 parts: (*1*) Initial method modifications incorporated certain quality control steps, including improved calibration procedures and control of the amount of material loaded on the GPC column by determining total residue of each sample. (*2*) An interlaboratory study was performed to evaluate the modified GPC method for separation of semivolatile pollutants from both high molecular weight soil and sediment components and late-eluting elemental sulfur. (*3*) The effect of dissolving extracts in 1:1 butyl chloride before processing by the modified GPC method was evaluated as a way to increase recovery of semivolatile analytes from waste and high concentration soil samples. (*4*) Cleanup of PCB-containing waste oils using the modified GPC method was evaluated for 2 reclaimed waste oil samples before PCBs were determined by GC analysis with electron-capture detection (ECD).

Experimental

Apparatus and Materials

(a) *Glassware.*—Erlenmeyer flask, 400 mL; centrifuge bottle, 200 mL, with Teflon-lined screw cap; borosilicate glass culture tubes, 13 × 100 mm, with Teflon-lined septa and hole screw cap; Kuderna-Danish (K-D) flask, 500 mL, with 10 mL concentrator tube and 3-ball macro-Snyder column; glass GPC column, 2.5 × 60 cm, with Teflon-lined support plungers (Analytical Biochemistry Laboratories, Columbia, MO), 2 L reservoir. Glassware was precleaned by washing with detergent and water, rinsing with distilled water, and heating in 475°C muffle furnace for 6 h before use.

(b) *Sonicator.*—Model W-375 (Heat Systems Ultrasonics, Inc.), output 10, duty cycle 50%, pulsed mode.

(c) *Centrifuge.*—Model K, International Equipment Co., operated at 2000 rpm.

(d) *Filters.*—0.45 and 0.5 μm (Millex-SR, Millipore, Bedford, MA).

(e) *Water bath.*—Model 1120A-1, Blue M Magni Whirl, 65-70°C.

(f) *Microbalance and weighing dishes.*—Mettler ME-30 or equivalent, capable of accurately weighing to 0.001 mg. Aluminum foil micro weighing dishes, Cahn No. 1568-02 or equivalent.

(g) *GPC system.*—Automated GPC apparatus assembled

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[1] Present address: Babcock & Wilcox, 1562 Beeson St, Alliance, OH 44601.

from ISCO ISIS autosampler (Lincoln, NE), 5 mL loop sampling module (O.I. Corp., College Station, TX), Rainin Rabbit HPX pump (Woburn, MA), and Altex (Deerfield, IL) Model 150 UV detector (254 nm) with biochemical flow cell (10 mm path length, 100 μL volume). Valve sequencing controlled by microprocessor assembled at Battelle Columbus Division. Flow rate of 5 mL/min. Operating pressures of 6–9 psi. To avoid drying of GPC column between periods of operation, fresh solvent was cycled through column when not in use. Chromatographic data were processed with Hewlett-Packard 1000 Series computer and Beckman (Waldwick, NJ) PeakPro software.

(h) *GPC column.*—2.5 cm × 60 cm glass column packed with 70 g Bio-Beads S-X3 (Bio-Rad, Richmond, CA, product obtained from Analytical Biochemistry Laboratories) in methylene chloride–cyclohexane (25 + 75) (to reduce gel swelling). After column was packed, methylene chloride was pumped through column at 5 mL/min for at least 2 h before column was connected to UV detector.

(i) *GC/MS system.*—Carlo Erba 4160 GC interfaced with Finnigan 3200 MS and operated in electron impact mode. Injector and transfer line temperatures 300°C; source temperature 180°C. A mass range of 40 to 450 amu was scanned in 0.75 s. Equipped with 30 m DB-5 fused-silica capillary column (J & W Scientific, Rancho Cordova, CA) with helium carrier gas flow rate of 30 cm/s. Injection: 2 μL splitless for 1.0 min. GC column temperature program: initial temperature, 100°C (hold for 4 min); 20°/min to 160°C (hold for 3 min); 3°/min to 300°C.

(j) *GC/ECD system.*—Hewlett-Packard 5890 GC equipped with electron-capture detector. Capillary column: 30 m DB-5 fused-silica column (J & W Scientific) with helium carrier gas flow rate of 30 cm/s. Injector temperature: 300°C. Injection: 2 μL splitless, hold for 45 s. GC column temperature program: initial temperature, 60°C (hold for 2 min); 8°/min to 300°C (hold for 10 min).

Reagents

(a) *Solvents.*—Pesticide grade methylene chloride, cyclohexane, butyl chloride, and acetone (Burdick and Jackson, Muskegon, MI).

(b) *Sodium sulfate.*—anhydrous (J. T. Baker, Phillipsburg, NJ). Heat in 475°C muffle furnace for 6 h.

(c) *Analytical standards.*—Polystyrene (Dow Styron 865D) was obtained from General Polymers, Columbus, OH. Mazola corn oil was used for calibration mixture. All other compounds used for GPC calibration and sample preparation were obtained from USEPA Repository of Toxic and Hazardous Materials (Research Triangle Park, NC) and used without further purification. Stock solutions of study analytes were prepared in 0.1–2.5 mg/mL solutions in methylene chloride. GPC calibration solution containing 1.0 mg/mL of polystyrene, 48 mg/mL of corn oil, 1.6 mg/mL of bis(2-ethylhexyl)phthalate, 0.028 mg/mL of 2,4-dinitrophenol, 0.17 mg/mL of 4-nitrophenol, 0.020 mg/mL of perylene, and 0.081 mg/mL of sulfur was prepared in methylene chloride. PCB-specific GPC calibration standard was prepared containing 0.1 mg/mL of Aroclor 1221 and 0.2 mg/mL of Aroclor 1260. Phenanthrene-d10 and chrysene-d12 were acquired from MSD Isotopes (Montreal, Canada), and GC/MS spiking solution was prepared at 500 and 670 μg/mL in methylene chloride.

Sample Collection

Initial method modification/interlaboratory study.—Several soil and sediment samples were used during this study. Soil was collected from one author's garden and from an Illinois site (ORD) where electrical capacitors were manufactured from the late 1940s to 1978. A saltwater sediment (NBH) was obtained from the Inner Harbor of New Bedford, MA, and a freshwater sediment (GLSM) was obtained from Grand Lake Saint Marys near Celina, OH.

A relatively large quantity of each sample was air-dried and sieved through a 2.5 mm screen. After the fines were thoroughly mixed by stirring and rolling, aliquots of each soil and sediment were weighed into glass bottles with Teflon-lined screw caps. All samples except the garden soil were known to be contaminated with PCBs; the Illinois soil also contained chlorinated naphthalenes. Previous GC/MS analyses had shown that an extract of a 50 g aliquot of each soil or sediment contained no detectable quantity of any analyte of interest except 2 phthalates known to be common laboratory contaminants.

For the interlaboratory study, 10 g Illinois soil and 20 g garden soil were combined to provide a sample containing approximately the desired total PCB concentration, based on results of previous analyses. An aliquot of an acetone solution containing other analytes of interest was added to each weighed sample aliquot to produce concentrations of 0.7–4 μg/g of each compound. A dichloromethane solution containing 0.10–0.27 μg/mL of each individual compound and 4 μg/mL of total PCBs (1.3 μg/mL of Aroclor 1016 and 2.6 μg/mL of Aroclor 1254) was provided to each laboratory for GPC processing, but solution composition was unknown to participating laboratories.

Effect of dissolving extracts in 1 + 1 butyl chloride–methylene chloride.—A black petroleum waste sludge, W2, was obtained from Paul Marsden of S-Cubed (San Diego, CA); high concentration soil samples No. 5 and 3313B-3 were obtained from Dorothy Lane of Western Research Institute (Laramie, WY). The 2 soil samples were collected at a Superfund waste site and contained high concentrations of petroleum hydrocarbons.

Cleanup of PCB-containing waste oils.—Two reclaimed waste oil samples, 149 and 183, were obtained from a Midwestern oil reclamation operation. Reclaimed waste oil 149 was a black sludge, partially soluble in methylene chloride. Reclaimed waste oil 183 was a brown, viscous oil, which was readily soluble in methylene chloride but difficult to filter and was reported to contain mineral oil (93–95%), lard oil (1–3%), chlorinated paraffin (1–3%), and sulfurized hydrocarbons (2.5% with 38% sulfur content). Both samples contained components which gave strong GC/ECD signals in the Aroclor 1260 region and prevented determination of PCBs by GC/ECD.

Sample Extract Preparation

Initial method modification/interlaboratory study.—Thirty g soil or sediment was mixed with 60 g anhydrous sodium sulfate and extracted by sonication (3 min) with three 100 mL portions of methylene chloride–acetone (1 + 1). The resulting extracts were combined and centrifuged for 15 min at 2000 rpm. The supernatant liquid was transferred to a Kuderna-Danish (K-D) apparatus, concentrated to 1 mL in a 65–70°C water bath, and diluted to 10 mL with methylene chloride. Each extract was filtered through a 0.5 μm filter and transferred to a culture tube sealed with a Teflon-lined septa and hole screw cap.

Effect of dissolving extracts in 1 + 1 butyl chloride–methylene chloride.—A modification of the USEPA "Draft High Concentration Protocol" (10) was used for sample preparation. The protocol is designed for processing waste

and soil samples containing high concentrations of analytes (usual sample size extracted is 1 g). A 14 g sample was mixed with 28 g sodium sulfate and 50 mL methylene chloride. The slurry was sonicated for 2 min and filtered through Pasteur pipets packed with 2–3 cm of muffled glass wool. The samples were filtered through 5 and 0.45 μm filters, and concentrated to < 50 mL using a K-D apparatus. The resulting extracts were spiked with sufficient amounts of 33 semivolatile organic compounds (see below) to achieve a concentration of 150 ppm for each analyte in the original sample. Six 5 mL portions (each corresponding to 1 g of sample extracted) of each extract were prepared; 3 portions were diluted to 10 mL with methylene chloride (final solvent composition 100% methylene chloride), and 3 portions were diluted to 10 mL with butyl chloride (final solvent composition 1 + 1 butyl chloride–methylene chloride) before GPC processing (see below).

Cleanup of PCB-containing waste oils.—One g of each waste oil was diluted to 10 mL with methylene chloride and filtered through 0.45 μm filters before GPC processing (see below).

GPC Processing of Sample Extracts

Initial method modification/interlaboratory study.—Samples were injected into the GPC system by way of the automated 5 mL loop sampling module and microprocessor control. Methylene chloride flow rate was 5 mL/min and run duration was 60 min. The GPC calibration solution was injected to determine the analyte collection fraction (fraction 2). Eluate collection was programmed to begin just before elution of bis(2-ethylhexyl)phthalate and continue until the peak minimum between perylene and sulfur.

Sample extracts were processed by GPC, and 3 fractions were collected for each sample. Fraction 1, which contained high molecular weight components, was collected from 10 min to the beginning of fraction 2, and fraction 3, which contained sulfur, was collected from the end of fraction 2 to 60 min. Fractions 1 and 3 were discarded. After all sample extracts were processed, the calibration solution was processed again to verify that the GPC column performance had remained constant. For the interlaboratory study, fraction 2, which contained the analytes of interest, was concentrated to 1 mL and spiked with 30 μL of a solution containing the internal standards (phenanthrene-d10 and chrysene-d12) before GC/MS analysis.

Effect of dissolving extracts in 1 + 1 butyl chloride–methylene chloride.—GPC calibration and processing were achieved as described above. Calibration results were identical for standards dissolved in 100% methylene chloride and in 1 + 1 butyl chloride–methene chloride. Triplicate injections of unspiked sample extracts and unspiked sample extracts dissolved in 100% methylene chloride and in 1 + 1 butyl chloride–methylene chloride were processed. For all samples, fraction 2 (28.0–49.4 mL, final volume of ca 105 mL), which contained the analytes of interest, was concentrated to 10 mL by using a K-D apparatus and then further concentrated to 1 mL by using a dry, purified stream of nitrogen. For recovery determinations, triplicate 5 mL aliquots of the study analytes, without matrix, in 1 + 1 butyl chloride–methylene chloride and in 100% methylene chloride were diluted to 105 mL with methylene chloride and then concentrated in the same manner as samples processed by GPC. Before GC/MS analysis, all extracts were spiked with 10 μL of a 100 μg/mL solution containing the internal standards phenanthrene-d10 and chrysene-d12.

Cleanup of PCB-containing waste oils.—The first step in processing the reclaimed waste oil samples was to perform an initial separation of PCBs from the higher molecular weight components and, if present, sulfur. To achieve this, the extracts were processed using the calibration procedure described above; fraction 2 (27.6–49.2 min) was collected and concentrated to a final volume of 1.0 mL as described above. Small portions of the resulting extracts (ca 100 μL) were diluted with hexane and reserved for subsequent analysis by GC/ECD.

A second, PCB-specific GPC cleanup step was performed as follows: A PCB-specific fraction was determined by processing a PCB calibration standard containing 0.1 mg/mL of Aroclor 1221 and 0.2 mg/mL of Aroclor 1260. The Aroclor mixture eluted between 34.7 and 45.8 min. To accommodate minor chromatographic variations, the collect fraction was set at 34.5–46.0 min. The reclaimed oil extracts were then reprocessed by the GPC method. The collected fraction for each of the 2 reclaimed waste oils was concentrated to 1.0 mL as described above. Hexane dilutions of the final extracts were prepared for GC/ECD analysis.

Residue Weight Determination

The nonvolatile residue of each of the starting extracts and collected fractions was determined by placing a filtered 100 μL aliquot of each solution into a tared aluminum micro weighing dish and placing the dish 8 ± 0.5 cm from a 250 watt heat lamp (General Electric). The evaporation surface was held at 80–100°C. After heating for 1.0 min, the foil cup was immediately transferred to the microbalance, weighed, and returned to the evaporation surface for an additional 30 s. Weights were recorded at 1.0, 1.5, and 2.0 min after the sample was placed under the lamp.

Capillary Gas Chromatography/Mass Spectrometry

Interlaboratory study/effect of dissolving extracts in 1 + 1 butyl chloride–methylene chloride.—Two μL of the calibration solutions and extracts were injected into the GC system using 1.0 min splitless injection. The temperature program described under *Apparatus and Materials* (i) was used for all analyses. Initial calibration was performed at 5 analyte concentrations (2.5, 5, 10, 20, and 50 ng/μL), keeping phenanthrene-d10 and chrysene-d12 concentrations constant at 15 and 20 ng/μL, respectively. Successful calibration required calculated relative standard deviations (RSD) of ≤ 30% for the study analyte response factors (RF) and mean RF exceeding 0.05. Calibration with the 10 ng/μL analyte solution was performed daily and each RF was calculated. Each daily RF was required to be within ± 25% of that obtained during initial calibration. The analytes were quantified using the integrated peak area of one characteristic ion. PCBs were quantitated by level of chlorination (total peak areas for each isomer group), using special software developed by USEPA (11).

Capillary Gas Chromatography/Electron-Capture Detection

Cleanup of PCB-containing waste oils.—Two microliters of Aroclor standards and extracts were automatically injected into the GC system using a 45 s splitless injection. The temperature program described under *Apparatus and Materials* (j) was used for all analyses. Final extracts diluted in hexane were used for analysis. Standards of Aroclors 1016, 1221, 1232, and 1242 at 1 μg/mL in hexane were chromatographed to serve as references for Aroclor identification. Aroclor concentrations were estimated by comparison of peak areas for 10 peaks in the samples with the corresponding peaks in the Aroclor standard.

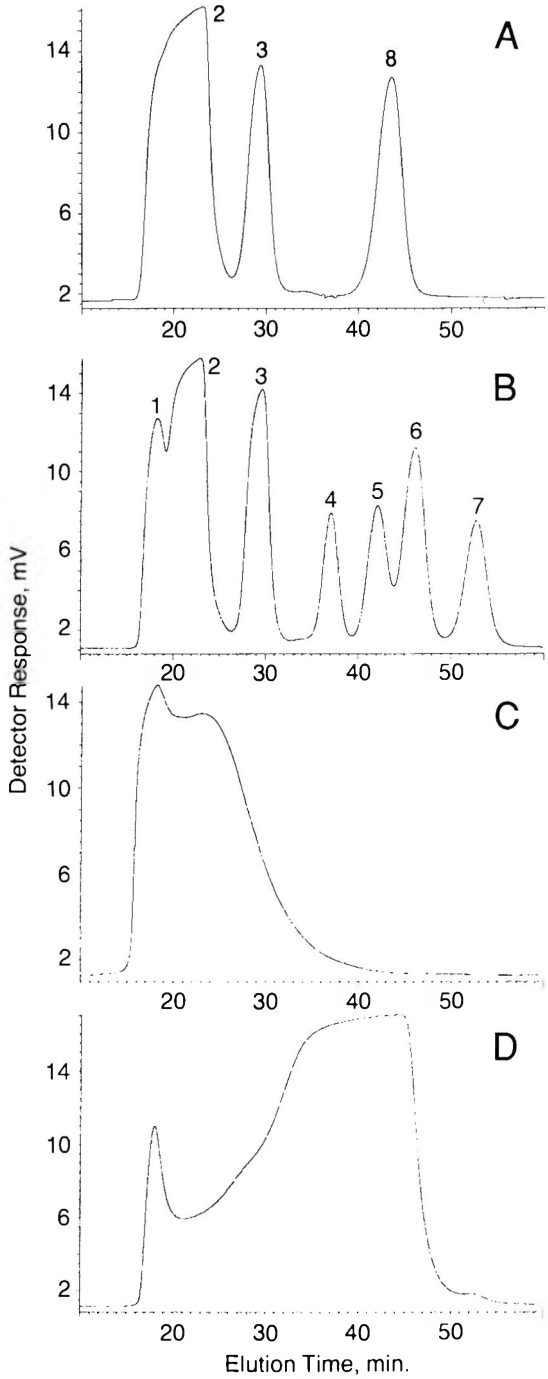

Figure 1. UV detector chromatograms obtained on 70 g S-X3 GPC column with 100% methylene chloride mobile phase at 5 mL/min: A, USEPA GPC calibration standard used in present GPC procedures (8–10); B, GPC calibration standard developed for this study; C, extract from 1 g sample of petroleum waste sludge W2 which appears to contain mainly high molecular weight (early eluting) material; and D, extract from 1 g sample of high concentration soil sample No. 5 which appears to contain both high and low molecular weight material. Peaks identities are: 1 = polystyrene (molecular weight 300 000); 2 = corn oil; 3 = bis(2-ethylhexyl)phthalate; 4 = 2,4-dinitrophenol; 5 = 4-nitrophenol; 6 = perylene; 7 = sulfur; 8 = pentachlorophenol.

Results and Discussion

Initial Method Modification

The first goal of the study was to optimize GPC calibration procedures for the 100% methylene chloride mobile phase. Analytes of interest were processed by the GPC method to determine elution volumes. The first eluting compound classes were phthalates and organophosphorus pesticides. The last eluting compound class in methylene chloride was the PAHs; benzo(ghi)perylene was the last eluting PAH measured. Figures 1A and 1B show chromatograms of the GPC calibration standard used in the present USEPA method (6, 7) (1A) and a calibration standard developed for this study (Figure 1B). For the present USEPA calibration protocol, a fraction (approximately 140 mL) is collected to include from ≥ 85% of the bis(2-ethylhexyl)phthalate until 10 min after the elution of pentachlorophenol (in Figure 1A, 28.0–53.0 min).

For the new calibration standard (Figure 1B), we deleted one compound and added 5 compounds to the calibration standard. The components added to the standard were polystyrene (molecular weight 300 000), 2,4-dinitrophenol, 4-nitrophenol, perylene, and sulfur. Polystyrene, a totally GPC-excluded component, serves as a marker for the determination of relative retention volume. Pentachlorophenol was deleted from the standard because it does not serve as a specific marker for the last eluting compounds, and it created waste disposal problems. 4-Nitrophenol and 2,4-dinitrophenol were used to monitor column resolution. Perylene, one of the last eluting compounds from the GPC column, was added to indicate the end of the collected fraction. (The last eluting analyte of interest, benzo(ghi)perylene, was not used because of its toxicity.) Sulfur was added to the calibration standard to allow separation of sulfur from the compounds of interest. With this new calibration solution, a fraction (approximately 115 mL) was collected from the beginning of the elution of bis(2-ethylhexyl)phthalate to the minimum between the perylene and sulfur peaks (in Figure 1B, 28.0–49.5 min). This modified calibration has 2 advantages over the present USEPA calibration procedure: (*1*) The fraction containing the analytes of interest is smaller in volume and thus required less time to be reduced to the final extract volume. (*2*) Sulfur is completely separated from the analytes of interest, and subsequent sulfur removal steps are eliminated. (Sulfur removal is often required for sediment and PCB-containing oil samples.)

Examples of GPC UV chromatograms for a petroleum waste sludge W2 and high concentration soil No. 5 are shown in Figure 1C and 1D. Both samples contained high molecular weight (totally excluded) components (ca 17 min). Sediments typically contained a prominent sulfur peak. The first fraction collected for soil/sediment extracts was usually colored (this fraction contained naturally occurring pigments).

To determine if sample components were irreversibly adsorbed to the column during GPC processing, all GPC eluates (3 fractions) were collected for 3 soil and sediment samples in duplicate. The total residue weight of each extract applied to the GPC column was compared with the sum of the residues of the 3 collected fractions (Table 1). The total residue weight before and after GPC separation compared well; thus, irreversible adsorption was not significant. However, the gradual darkening of the GPC column after processing several extracts was likely caused by adsorption of small quantities of dissolved materials or filterable (< 0.5 μm) particulate materials.

The capacity of the 70 g S-X3 column was determined by processing a mixture of soil and sediment extracts containing 20, 200, 400, and 900 mg material per injection. The analytes of interest were always separated from sulfur, and the retention volumes were not affected. Normally, we injected no more than 0 5 g material (monitored by measuring the residue weight of each extract). Typically, less than 100 mg material was present in methylene chloride extracts of 30 g samples of soils, waste sludges, and sediments and capacity was not exceeded. For oily waste samples, or soils containing

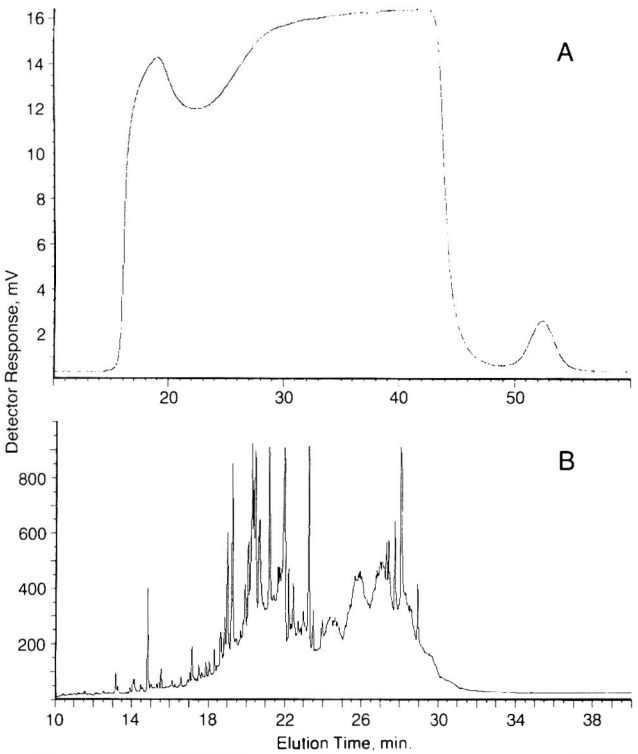

Figure 2. Chromatograms of waste oil sample 149: A, UV detector GPC chromatogram for the first GPC processing, using run conditions described in *Apparatus and Materials*. Sample apparently contains both high molecular weight (early eluting) material and elemental sulfur (ca 52 min). Fraction was collected from 27.6 to 49.2 min. B, GC/ECD detector chromatogram of collected fraction from first GPC processing. GC/ECD run conditions are given in *Apparatus*. Interference peaks at 18–30 min prevented determination of PCB.

other wastes, however, methylene chloride extractable material was significantly higher, and the column capacity may be exceeded for soils containing other wastes, such as oils. High residue sample extracts can be diluted and processed in 2 or more GPC injections, and the resulting collected fractions can be recombined prior to analysis. Replicate GPC processing can be used for cleanup of difficult samples such as waste oils (see below).

Interlaboratory Study

Recoveries of semivolatile pollutants in solutions processed with the GPC calibration procedure developed in this study were measured in a 5 laboratory study. The method included the addition of quality assurance procedures such as daily calibration of the GPC column, controlling the amount of material applied to the column, and monitoring of sample UV chromatograms to be sure that the correct fraction was collected.

Each laboratory processed, by GPC, 2 aliquots of a solution containing each analyte at a concentration of 0.1–0.3 μg/mL and a total PCB concentration of 4 μg/mL (as 1.3 μg/mL of Aroclor 1016 and 2.6 μg/mL of Aroclor 1254). The results of the interlaboratory study for the solution are given in Table 2. With the exception of 4-nitroaniline, analyte recoveries were 87–112% with RSDs of 6.7–26%. The reported high recovery of 4-nitroaniline may be caused by its poor chromatographic peak shape, which hampers accurate peak measurement. The mean recovery of 6 pesticides for the 5 laboratories was 100% (range of 91–114%) with a mean RSD of 16% (range of 6.6–33%). The interlaboratory study also involved GPC processing of soil sample extracts. Duplicate 30 g aliquots of soil sample containing environmentally introduced PCBs and spiked with 0.7–4 μg/g of the other study analytes were extracted. After extracts were concentrated and processed with the GPC method, each concentrated GPC eluate was analyzed by GC/MS.

Table 1. Nonvolatile residues of soil and sediment extracts before and after gel permeation chromatographic cleanup

Sample	Total residue weight, mg[a]	
	Before cleanup	After cleanup
NBH-1	14	14
NBH-2	14	13
GLSM-1	4.7	4.4
GLSM-2	4.1	3.5
ORD-3-1	1.8	1.7
ORD-3-2	2.5	2.7
Method blank	—	—

[a] Determined for 6.25 g soil/sediment extracted. Total residue for 30 g soil/sediment extracted, as specified in USEPA protocol (8, 9), would be higher.

This method performance can be compared with that obtained in a previous interlaboratory study that used the USEPA GPC method (8) for a soil sample from the same site. That soil sample was fortified with 8 pesticides (6 unlabeled and two ^{13}C-labeled compounds) at concentrations of 2–4 μg/g. Duplicate 10 g samples were analyzed by 6 laboratories (12). Mean recovery of pesticides for the 6 laboratories was 58% (range of 28–83%) with a mean RSD of 43% (range of 31–53%). For the 2 compounds common to both studies, accuracy and precision of measured concentration were improved by using the modified GPC method. Mean recovery of beta-hexachlorocyclohexane (beta-BHC) increased from 61 to 95% and mean RSD improved from 46 to 12%; for gamma-BHC, mean recovery increased from 56 to 91%, and mean RSD improved from 42 to 16%. For total PCBs, no true value was known, but mean RSD for measured concentrations was 38% in the earlier interlaboratory study and 8.9% in this study. The modified GPC method was also successfully used

Table 2. Recovery of semivolatile organic pollutants after GPC cleanup for solution processed in interlaboratory study

Compound	Amount spiked, μg	Mean Recovery,[a] %	Mean RSD,[a] %
4-Nitroaniline	1.33	190	34
Phenol-d5	0.667	100	11
2-Fluorophenol	0.667	87	26
2,4,6-Trichlorophenol	1.00	91	11
2,4,6-Tribromophenol	1.00	89	23
Nitrobenzene-d5	1.00	96	13
1,2,4-Trichlorobenzene	0.833	97	11
4-Chlorophenylphenyl ether	0.833	99	13
2-Fluorobiphenyl	0.667	100	9.3
Terphenyl-d14	1.00	100	25
Butylbenzylphthalate	0.667	110	9.1
Benzo(a)pyrene	0.833	98	6.7
Benzo(ghi)perylene	1.33	99	23
DDT	0.667	91	15
gamma-BHC	0.667	91	8.9
Endrin	0.500	100	25
Aldrin	0.500	94	11
Endrin ketone	1.00	90	13
gamma-Chlordane	0.667	97	10
Total PCBs[b]	19.7	110	11

[a] Mean recovery and RSD were calculated from mean of duplicate measurements of each analyte reported by the 5 laboratories.

[b] Mixture of Aroclors 1016 (6.67 μg) and 1254 (13.0 μg).

Table 3. Effect of GPC processing of samples dissolved in 1 + 1 butyl chloride–methylene chloride or 100% methylene chloride on recoveries of study analytes[a]

	Petroleum waste sludge W2				High concentration soil No. 5				High concentration soil 3313B-3			
	BuCl/MeCl[b]		MeCl[c]		BuCl/MeCl		MeCl		BuCl/MeCl		MeCl	
	Mean % rec.[d]	RSD, %	Mean % rec.	RSD, %	Mean % rec.	RSD, %	Mean % rec.	RSD, %	Mean % rec.	RSD, %	Mean % rec.	RSD, %
2-Methylphenol	102	5.3	93	2.8	79	6.1	97	6.8	89	15	92	7.9
4-Methylphenol	108	8.8	98	3.6	82	6.1	93	6.6	96	16	98	9.0
2,4-Dinitrophenol	136	14	130	0.91	94	19	112	10	130	22	130	30
4-Chlorophenol	100	10	96	3.0	78	7.2	92	6.6	85	29	98	7.1
2,4,6-Trichlorophenol	93	6.2	84	2.0	82	6.9	93	3.5	83	13	87	4.9
Pentachlorophenol	122	3.7	124	2.6	78	13	100	5.0	100	30	110	17
1,2-Dichlorobenzene	95	7.4	90	7.0	82	8.0	100	9.1	79	20	94	7.3
1,2,4-Trichlorobenzene	93	7.1	86	2.4	79	7.9	96	4.1	75	21	90	4.7
1,2,3,4-Tetrachlorobenzene	89	7.4	82	1.0	80	6.6	94	6.1	72	21	88	4.8
Pentachlorobenzene	88	5.5	83	1.5	81	8.3	92	6.6	70	21	85	4.3
Hexachlorobenzene	90	4.1	83	1.7	80	7.4	91	5.1	69	23	83	3.8
Nitrobenzene	123	7.8	107	2.3	85	10	95	11	110	9.6	100	11
4-Chlorophenyl-phenylether	100	1.3	83	1.8	92	11	120	8.5	93	8.9	90	4.5
1,2-Dibromo-3-chloropropane	98	5.2	93	3.8	81	7.6	100	9.0	86	9.6	93	5.6
Aniline	81	6.9	80	2.3	33	18	45	14	60	18	69	2.6
N-Nitroso-di-*n*-butylamine	103	14	96	4.4	110	7.1	127	2.9	99	12	100	20
Dimethoate	112	5.6	103	4.8	92	3.6	107	13	110	6.8	110	6.2
Famophos	100	2.2	93	4.8	118	16	108	7.1	110	4.5	100	5.7
Temephos	997	230	786	161	210	52	107	77	420	19	300	180
Phorate	104	5.8	95	1.5	82	6.2	82	8.0	100	12	81	12
Isophorone	112	5.6	100	4.1	87	8.2	100	12	110	4.8	110	6.0
Diethylphthalate	94	4.7	87	1.6	88	6.1	101	5.7	93	6.1	96	6.0
Butylbenzylphthalate	99	3.4	93	2.9	107	18	111	9.9	99	7.8	97	5.8
Bis(2-ethylhexyl)phthalate	90	5.3	85	4.3	98	14	111	8.2	90	6.4	88	3.1
Di-*n*-butylphthalate	106	3.9	97	3.0	113	17	116	8.8	100	6.6	100	5.2
Naphthalene	99	6.6	92	3.1	84	8.5	107	9.2	88	15	110	4.8
2-Methylnaphthalene	95	6.5	86	1.9	85	8.8	100	9.2	89	8.7	98	5.1
Fluorene	96	5.5	85	1.9	89	7.5	98	5.2	92	6.5	95	4.0
Pyrene	97	2.7	91	1.7	104	20	109	8.1	80	23	93	5.1
Benzo(a)anthracene	95	3.4	88	1.5	107	16	110	8.1	82	18	91	4.4
Benzo(a)pyrene	90	2.3	84	0.94	93	14	96	1.3	76	22	86	5.4
Perylene	93	1.8	86	3.2	100	16	100	3.7	75	23	89	6.0
Benzo(ghi)perylene	89	1.5	89	5.3	102	19	100	12	77	21	94	5.0

[a] GPC processing of samples representing 1 g extracted material with analytes spiked at 150 ppm.
[b] Five mL samples loaded onto GPC in 1 + 1 butyl chloride-methylene chloride.
[c] Five mL samples loaded onto GPC in 100% methylene chloride.
[d] Mean and RSD calculated from 3 determinations.

to separate the study analytes from high molecular weight soil components and elemental sulfur. No additional sulfur removal step was required.

Effect of Dissolving Extracts in 1 + 1 Butyl Chloride–Methylene Chloride

Previous workers have suggested that dissolving extracts for GPC processing in 1 + 1 butyl chloride–methylene chloride rather than 100% methylene chloride increases the recovery of semivolatile analytes (13). The GPC mobile phase is unchanged except for the presence of 2.5 mL butyl chloride (for a 5 mL sample volume injected). The increase in recovery was thought to be most notable for "high concentration" samples, perhaps because the butyl chloride increased the solubility of the high level analytes. Thus, we investigated the possible effect of dissolving extracts in 1 + 1 butyl chloride–methylene chloride on analyte recoveries for 3 samples processed by the Draft High Concentration Protocol (10).

The recoveries of analytes for the 2 sample introduction solvents (1 + 1 butyl chloride–methylene chloride and 100% methylene chloride) were determined by triplicate processing of spiked samples of petroleum waste sludge W2, high concentration soil No. 5, and high concentration soil 3313B-3. Recoveries were calculated relative to triplicate analyses of standard solutions prepared with either butyl chloride–methylene chloride or 100% methylene chloride, as appropriate, and were corrected for any detectable quantities of analytes determined from triplicate analyses of unspiked samples.

GPC chromatograms of 2 sample extracts (W2 and No. 5) dissolved in methylene chloride are shown in Figure 1. To evaluate the efficiency of the GPC cleanup for the 3 samples studied, the total residue of each of the 3 samples before and after GPC processing was determined. Petroleum waste sludge W2 showed efficient removal of most (94%) of the high molecular weight material (Figure 1). The amount of material removed by GPC cleanup for the high concentration soil sample No. 5 was small (ca 12%), and consistent with the elution of a large amount of material during the collected fraction (see Figure 1). High concentration soil sample 3313B-3 showed efficient cleanup (84% removal).

The recoveries of analytes for petroleum waste sludge W2, high concentration soil No. 5, and high concentration soil 3313B-3 are given in Table 3. For petroleum waste sludge W2, 13 analytes (2,4,6-trichlorophenol, hexachlorobenzene, 4-chlorophenylphenyl ether, famophos, phorate, isophorone, butylbenzyl phthalate, di-*n*-butyl phthalate, fluorene, py-

rene, benzo(a)anthracene, benzo(a)pyrene, and perylene) gave higher recoveries when sample was introduced in 1 + 1 butyl chloride–methylene chloride. Still, absolute differences in measured recoveries were small (ca 5–10%). For most compounds, differences in the measured recoveries of the study analytes for the 2 sample introduction solvents did not exceed the relative standard deviation for the 3 injections. These results do not show significant improvement for GPC sample introduction in 1 + 1 butyl chloride–methylene chloride, but may not be applicable to other waste sludge matrixes.

In a separate study, 11 of the study analytes were determined in petroleum waste W2, using sample introduction in 1 + 1 butyl chloride–methylene chloride and 100% methylene chloride (13). The analytes in common to both studies were 2,4,6-trichlorophenol, hexachlorobenzene, nitrobenzene, 4-chlorophenylphenyl ether, 1,2-dibromo-3-chloropropane, aniline, *N*-nitroso-di-*n*-butylamine, isophorone, butylbenzyl phthalate, 2-methylnaphthalene, and pyrene. The relative recoveries of all of the analytes common to both studies, with the exception of hexachlorobenzene, aniline, and pyrene, were within 20% of each other. However, the recoveries of hexachlorobenzene, aniline, and pyrene were reported in the previous study (13) to be improved by greater than 50% with sample introduction in 1 + 1 butyl chloride–methylene chloride. These results were not obtained in the present study; small increases in recoveries (7 and 6% for hexachlorobenzene and pyrene, respectively) or no increased recoveries (for aniline) were observed for samples introduced in 1 + 1 butyl chloride–methylene chloride.

For the high concentration soil No. 5, the recoveries of several analytes were slightly higher with sample introduction in 100% methylene chloride (Table 3), but the relative differences in recoveries were small. For high concentration soil 3313B-3, no differences in recoveries were observed for the 2 sample introduction methods.

With the exception of aniline and temephos, the average study analyte recovery was 97% for 1 + 1 butyl chloride–methylene chloride sample introduction, 100% for 100% methylene chloride sample introduction, and 99% for both sample introduction methods. Aniline and temephos showed unusually low and high recoveries, respectively, in all samples. The low recovery of aniline may have been caused by its relative volatility or poor chromatographic performance. The low mass spectrometry sensitivity for temephos most likely resulted in errors.

The analytes appeared to show similar recoveries in the 3 matrixes studied, and thus there is no evidence of matrix-specific effects. Such effects have been reported, for example, for a carbon tetrachloride still bottom waste W4 (13). One would need to increase the number of samples investigated to provide a more thorough evaluation of possible matrix-specific effects.

Butyl chloride was observed to elute from 195 to 225 mL, which is in the collected fraction (determined by gas chromatography). Thus, the 2.5 mL butyl chloride used for sample introduction is present in the final collected extract is difficult to remove by Kuderna-Danish or nitrogen blow-down techniques during the final solvent reduction step to 1.0 mL.

The lack of consistent increase in recoveries of the analytes with sample introduction in 1 + 1 butyl chloride–methylene chloride suggested that the present GPC protocol should not be modified.

Cleanup of PCB-Containing Waste Oils

Another effort involved the evaluation of the GPC protocol for removal of hydrocarbon and sulfur interferences from 2 waste oil samples, waste oil 183, a finished reclaimed oil product, and waste oil 149, which contained solid waste generated from the reclamation process. Waste oil 149 was related to waste oil 183. These 2 samples were previously reported (to USEPA) to be difficult to analyze for PCBs by GC/ECD because of interfering compounds. The interferences were most likely long-chain hydrocarbons, possibly including "sulfurized hydrocarbons" which may produce an ECD response.

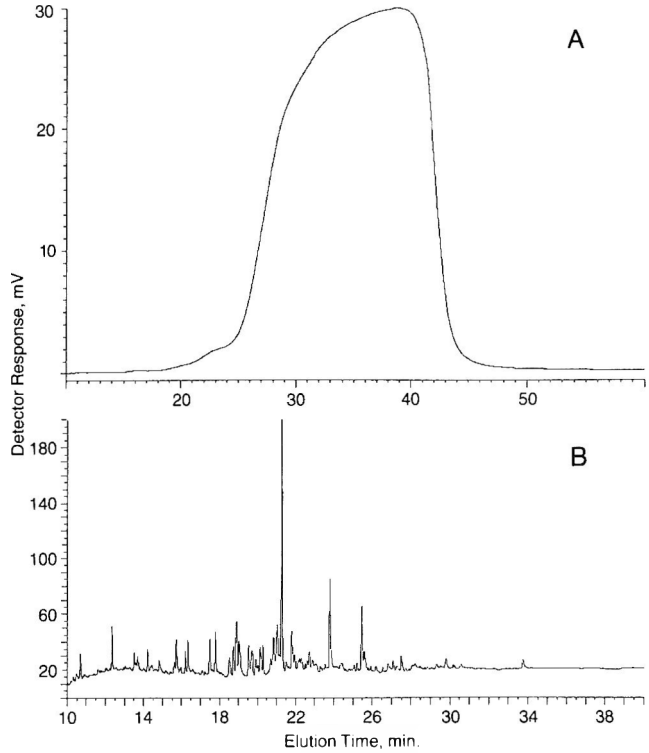

Figure 3. Chromatograms of waste oil sample 149: A, UV detector GPC chromatogram for second, PCB-specific GPC processing, using run conditions described in *Apparatus and Materials*. Sample no longer contains early eluting components or elemental sulfur. Fraction was collected from 34.5 to 46.0 min. B, GC/ECD detector chromatogram of collected fraction from second GPC processing. GC/ECD run conditions are given in *Apparatus*. Aroclor 1221 or 1232 is tentatively identified in sample.

Because a small sample (1 g) was required for PCB determinations, a modification of the Draft High Concentration Protocol (10) was used. The sample was initially processed using the GPC procedures described above. The collected fraction, 27.6 to 49.2 min, allowed the separation of a portion of the early eluting material and all of the sulfur from the analytes of interest. The sulfur was likely introduced with the sulfurized hydrocarbons, reported to be present at 2.5% of the finished oil product. The residue weights of the waste oil samples before and after GPC processing are given in Table 4. GPC processing of waste oil samples 149 and 183 removed 26 and 3.4% of the total residue, respectively.

For illustration, Figure 2A shows a GPC chromatogram of waste oil 149 for the first GPC processing. In Figure 2A, early eluting (high molecular weight) material and a late eluting peak corresponding to sulfur (52.5 min) are observed. A GC/ECD chromatogram of fraction 2 of waste oil 149 after the first GPC processing is shown in Figures 2B. Although several peaks are observed in the PCB region (15–33 min), no recognizable Aroclor pattern is observed. Several broad peaks observed in the chromatograms are most likely hydrocarbons not removed by the first GPC processing step.

Because it was not possible to determine PCBs as Aroclors in the 2 oil samples, a second GPC processing of the collected fractions from the first GPC processing was performed. This time, a PCB-specific GPC fraction was collected. To determine the PCB-specific fraction to be collected, a standard containing 0.1 mg/mL of Aroclor 1221 and 0.2 mg/mL of Aroclor 1268 was processed by the GPC method. Those 2 Aroclors were chosen to represent the range of PCB chlorination levels. The PCB-specific fraction was collected from 34.5 to 46.0 min.

Figure 3A shows the GPC chromatogram of waste oil sample 149 after the second (PCB-specific) GPC processing. Collection of a PCB-specific fraction allowed further separation of the early eluting material. Figure 3A shows that the sulfur interference was completely removed in the first cleanup step.

Residue weights of the waste oil samples were determined before and after each GPC processing step. The first GPC processing of waste oil samples 149 and 183 removed 26 and 3.4% of the total residue, respectively. The PCB-specific GPC processing of waste oil samples 149 and 183 removed 95 and 84% of the residue, respectively. The overall residue removed for the combined GPC cleanup steps was 98% for waste oil 149 and 90% for waste oil 183. We do not know if similar overall cleanup would have been achieved for a single, PCB-specific GPC processing. Our experience is, however, that repetitive GPC processing steps may be required for difficult sample matrixes such as oily wastes. Still, the method used here may be improved by collecting a smaller fraction during the first GPC processing.

A GC/ECD chromatogram of the waste oil 149 after the PCB-specific GPC processing is shown in Figures 3B. The oil hydrocarbon background peaks and several peaks that were present after 27 min in the initial GPC-processed extract (Figure 2B) are no longer present.

GC/ECD chromatograms of both waste oil samples most closely resembled Aroclors 1221 and 1232, but peaks not present in the Aroclor standards are observed. These peaks may be products of PCB degradation, residual sulfurized hydrocarbons, or other halogenated organics. Analysis by GC with a Hall detector would eliminate peaks produced by sulfur-containing components. Levels of PCBs as Aroclor were estimated by comparison of the peak height of the largest PCB peak (retention time ca 21 min) to the corresponding peak in the Aroclor standards. PCB levels in waste oil 149 and 183 were estimated to be 4 and 100 μg/g. Because the identification of PCBs as Aroclors in the 2 waste oil samples was not possible, PCB content should be confirmed by capillary GC/mass spectrometry (GC/MS).

Repetitive GPC processing of waste oil samples was shown to be successful for the determination of PCBs in 2 waste oils. Single GPC processing did not provide sufficient cleanup for determination of PCB by GC/ECD. Collection of an analyte-specific fraction removed hydrocarbon components and elemental sulfur and should provide superior cleanup of samples when the analytes of interest elute from the GPC column over a narrow volume.

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One-Step Sample Preparation Technique for Broad Spectrum Gas Chromatographic/Mass Spectrometric Determination of Organic Priority Pollutants in Water

ANGELO ALFIERI, GEORGE CRAWFORD, and IJAZ AHMAD
Ministry of the Environment, Laboratory Services Branch, Trace Organics Section, 125 Resources Rd, Rexdale, Ontario M9W 5L1, Canada

A rapid and cost-effective sample preparation technique is described for the qualitative and quantitative determination of a broad spectrum of organic contaminants in water. The technique involves simultaneous in-situ acetylation of phenols with acetic anhydride and one-step extraction of phenol acetates and basic and neutral organic compounds from water. The extract is concentrated and analyzed by gas chromatography/mass spectrometry. The recovery and precision data of selected base, neutral, and phenolic compounds are given. The advantages and disadvantages of the in-situ acetylation/extraction technique are discussed.

Contamination of the environment from agricultural chemicals, industrial activities, spills, and upsets is widely recognized. Contamination of water is a major concern. The health of all life forms is affected by these contaminants in water. The awareness and concern about the quality of the environment have increased during the last 15–20 years and, because of these concerns, scientists are involved in programs to assess water quality.

The Laboratory Services Branch of the Ontario Ministry of the Environment has developed a regulatory program which will require industries and municipal sewage treatment plants to monitor the presence of hazardous contaminants in their final effluent and, in some cases, influents and other process streams. To direct this monitoring, the Ministry has generated a priority pollutants list of hazardous chemicals representing a broad spectrum of organic and inorganic chemicals. The objective of the research described in the present paper was to develop a cost-effective protocol for the determination of organic priority pollutants (Table 1) in wastewater.

Gas chromatographic/mass spectrometric (GC/MS) methods (1–4) are the best methods for determining organic contaminants in wastewater. These methods consist of 2 parts, sample preparation and instrumental analysis. The autosampler provides dedicated automation for instrumental analysis. However, sample preparation, which involves extraction of 1 L water sample with organic solvent at basic and acidic pH followed by cleanup of the multifractions, is cumbersome. This technique, while useful for some applications and providing cleaner fractions for instrumental analysis, has several disadvantages: it is time consuming and labor intensive and reduces sample throughput; it may cause losses and variability of results due to the multi-step extraction and separation procedure; the instrumental cost for the GC/MS analysis is higher due to multifraction analysis; there is a potential for the rapid loss of performance of the gas chromatographic capillary column if underivatized acids/phenols are analyzed.

We describe a simple, one-step sample preparation technique for the determination of a wide range of organic contaminants in water by using extraction with in-situ acetylation. Application of the acetylation technique for determining selected phenols in aqueous solution has been documented by Coutts et al. (5) and other workers (6–17). However, the present work demonstrates the simultaneous acetylation of phenols and one-step extraction of acetylated phenols and base/neutral organic compounds (priority pollutants) from water.

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METHOD

Apparatus and Reagents

(a) *Gas chromatograph.*—Hewlett-Packard Model 5890 equipped with 5970 mass selective detector (MSD), series 300 computer ChemStation, split/splitless injection port, and Model 7673A autosampler. Capillary column: DB-5, 30 m × 0.25 mm id, 0.25 μm film (J & W Scientific, Inc., Rancho Cordova, CA 95670) directly interfaced to MSD ion source. Operating conditions; electron ionization (EI); electron energy 70 eV; selected ion monitoring mode and dwell time/ion 100 ms; temperature program: injection port 275°C, transfer line 300°C, column 75°C, hold for 3 min, increase to 120°C at 15°/min, then to 190°C at 5°/min, then to 305°C at 15°/min, and hold for 10 min. Helium carrier gas at 50 kPa.

(b) *Organic solvents.*—Pesticide grade or distilled-in-glass.

(c) *Organic-free water.*—Prepared by extracting distilled water with three 50 mL portions of dichloromethane.

(d) *Anhydrous sodium sulfate.*—Granular (Fisher Scientific Co.).

(e) *Acetic anhydride.*—AnalaR, 98+% (BDH Chemicals, Toronto, Ontario M8Z 1K5, Canada).

(f) *Potassium chloride.*—Analytical reagent (BDH Chemicals).

(g) *Base, neutral, and phenol standards.*—Analytical grade, 99+% purity (various suppliers). Prepare stock solutions in toluene at 1 mg/mL and store in dark at −20°C. Prepare working standards (mixtures) at appropriate concentrations and store in dark at 4°C.

Preparation of Spiked Samples

Add appropriate amount of standard mix solutions to water samples to produce desired concentration, or dilute solutions to produce test concentration.

Sample Collection, Preservation, and Handling

Follow U.S. Environmental Protection Agency guidelines (1).

Sample Preparation

To 500 mL sample in 1 L brown glass sample bottle, add 100 μL surrogate standard containing 10 μg chrysene-d_{12} and 10 μg 4-fluorophenol. Add 10 g potassium bicarbonate ($KHCO_3$) followed by 50 mL dichloromethane. Shake mixture, release pressure periodically by loosening the bottle cap, and then tumble 15 min.

To solution, add 2.5 mL acetic anhydride, shake gently until evolution of carbon dioxide subsides, and then tumble 30 min. Add 5 g potassium chloride and tumble again 30 min.

Remove dichloromethane layer with 25 mL pipet and collect extract in 250 mL Erlenmeyer flask. Repeat extraction with two 25 mL portions of dichloromethane.

Table 1. Priority pollutants: list of target organic contaminants

Base/neutral compounds	Phenolic compounds
Acenaphthene	4-Chloro-3-methylphenol
Acenaphthylene	2-Chlorophenol
Anthracene	*m*-Cresol
Benzo(a)anthracene	*o*-Cresol
Benzo(a)pyrene	*p*-Cresol
Benzo(b)fluoranthene	2,4-Dichlorophenol
Benzo(k)fluoranthene	2,6-Dichlorophenol
Benzo(g,h,i)perylene	2,4-Dimethylphenol
Bis(2-chloroethyl) ether	2,4-Dinitrophenol
Bis(2-chloroethoxy) methane	4,6-Dinitro-*o*-cresol
Bis(2-chloroisopropyl) ether	4-Nitrophenol
Bis(2-ethylhexyl) phthalate	Pentachlorophenol
4-Bromophenyl phenyl ether	Phenol
Butyl benzyl phthalate	2,3,4,5-Tetrachlorophenol
Camphene	2,3,4,6-Tetrachlorophenol
1-Chloronaphthalene	2,3,5,6-Tetrachlorophenol
2-Chloronaphthalene	2,3,4-Trichlorophenol
4-Chlorophenyl phenyl ether	2,4,5-Trichlorophenol
Chrysene	2,4,6-Trichlorophenol
Dibenzo(a,h)anthracene	3,4,5-Trichlorophenol
Di-*n*-butyl phthalate	
Di-*n*-octyl phthalate	
2,4-Dinitrotoluene	
2,6-Dinitrotoluene	
Diphenylamine	
Diphenyl ether	
Fluoranthene	
Fluorene	
Indeno(1,2,3-cd)pyrene	
Indole	
1-Methylnaphthalene	
2-Methylnaphthalene	
Naphthalene	
5-Nitroacenaphthene	
N-Nitrosodi-*n*-propylamine	
N-Nitrosodiphenylamine	
Perylene	
Phenanthrene	
Pyrene	

Dry combined extracts with 40 g anhydrous sodium sulfate. Add 2 mL isooctane and concentrate extract to ca 2 mL on rotary evaporator. Add 50 μL (10 μg) anthracene-d_{10} standard and dilute to 5.0 mL with isooctane. Inject 2 μL sample extract into GC/MSD system for analysis.

Results and Discussion

Using in-situ acetylation/extraction technique, all the base, neutral, and phenolic compounds listed in Table 1 were extracted in one fraction, which was analyzed without further cleanup using gas chromatography/mass spectrometry in electron impact-selected ion monitoring mode (GC/MS-EI-SIM). Identity of the compounds in the extract was confirmed by using retention time data and the relative abundance of the 3 characteristic masses (m/z) (Table 2). Compounds were quantitated by using an internal standard technique with a single primary characteristic ion.

Many phenol acetate standards were not commercially available for peak identification; therefore, all the phenols were acetylated under the conditions described in the experimental section to establish retention time and mass spectral data of the phenol acetates. Recovery data given in Table 3 were established using organic-free water. Relative recoveries were reported for all compounds. Method standard was used to calculate relative recoveries, which involved spiking 500 mL organic-free water with all the target compounds at an appropriate concentration and processing them along with the samples. The recoveries from the samples were then compared with that of the method standard. The accuracy and precision data were calculated from 8 recovery measurements at each of the three spike levels. The recovery and precision data of phenols were better than the data reported in the literature (1, 18, 19), and that obtained by solvent extraction of acidified water sample. The in-situ acetylation/extraction technique eliminated the drawback of low recoveries of some phenols obtained by solvent extraction of these compounds at acidic pH.

Method detection levels of all the compounds were calculated according to the Ontario Ministry of the Environment guidelines (20) and were reported in Table 2. The method was not suitable for trace residue analysis of 2,4-dinitrophenol and 4,6-dinitro-*o*-cresol. These compounds were not detected at a spike level of 100 ppb. It was primarily due to the mass spectrum fragmentation of the 4,6-dinitro-*o*-cresol and 2,4-dinitrophenol acetates in which all the m/z ions were less than 1% of the base peak. Figure 1 shows mass spectral fragmentation of 2,4-dinitro-*o*-cresol acetate and its parent phenol. Similar mass spectral data were obtained with 2,4-dinitrophenol and its acetate derivative. The comparison of the mass spectra in Figure 1 clearly demonstrated the cause of higher MDL for these 2 nitrophenols.

N-Nitrosodiphenylamine decomposed in the gas chromatograph's injection port and could not be separated from diphenylamine: therefore, an appropriate method, such as EPA 607, should be used if the determination of these 2 compounds is necessary. Benzo(b)fluoranthene and benzo(k)fluoranthene have identical mass spectral properties and co-eluted at 29.93 min. Indeno(1,2,3-cd)pyrene interfered with dibenzo(a,h)anthracene because of co-elution and similar ions in the mass spectra. The co-elution problem was overcome by simply changing the column temperature program between 190 and 305°C (increase from 190 to 285°C at 15°/min, increase to 290°C at 1°/min and hold for 5 min).

To demonstrate the use of this method, water samples were collected from a landfill site and were spiked with 8 selected compounds. The spiked and unspiked samples were processed using this method. The results are reported in Table 4. The recovery of each compound fell within the range established by EPA guidelines (1). Figure 2 is a total ion chromatogram of a wastewater sample that was processed using this method. The sample was collected from a plastic industry and submitted to this laboratory for target compounds analysis.

The advantages of the simultaneous acetylation/extraction technique are several: it is cost-effective because it involves simultaneous acetylation of phenols and extraction of base, neutral, and phenolic compounds in one fraction; GC/MS instrumental time is saved, because one run is required instead of 2 or more; and cleanup of the extract is rarely needed—fatty acids responsible for major interference in wastewater analysis remain in the discard phase.

There are also disadvantages: the technique is not suitable for carboxylic acids; the method is not applicable for trace residue analysis of 4,6-dinitro-*o*-cresol and 2,4-dinitrophenol; and the method is specifically designed for GC/MS-SIM analysis; however, gas chromatography with electron capture detection or flame ionization detection of the extract may lead to a complex chromatogram.

The application of this sample preparation technique to primary and secondary amines, speciation of phenols (e.g.,

Table 2. Retention times, method detection limits, and characteristic ions for organic priority pollutants

Compound	Retention time, min	Method detection limit, μg/L	Characteristic ions, m/z (rel. abundance)		
			Primary	Secondary	Secondary
Base/neutral compounds					
Camphene	4.59	3.5	121 (100)	79 (88)	136 (30)
Bis(2-chloroethyl) ether	5.06	4.4	93 (100)	65 (32)	95 (35)
Bis(2-chloroisopropyl) ether	6.10	2.2	121 (100)	77 (48)	123 (36)
N-Nitrosodi-*n*-propylamine	6.35	3.0	70 (100)	130 (26)	101 (10)
Bis(2-chloroethoxy) methane	7.73	3.5	93 (100)	95 (34)	123 (14)
Naphthalene	8.26	2.7	128 (100)	102 (12)	126 (9)
Indole	10.20	1.9	117 (100)	90 (58)	89 (40)
2-Methylnaphthalene	10.30	2.2	142 (100)	141 (82)	115 (35)
1-Methylnaphthalene	10.65	3.2	142 (100)	141 (84)	115 (37)
2-Chloronaphthalene	11.96	1.8	162 (100)	127 (50)	164 (36)
1-Chloronaphthalene	12.07	2.5	162 (100)	127 (50)	164 (36)
Diphenyl ether	12.48	5.3	170 (100)	141 (69)	115 (21)
Acenaphthylene	13.54	1.4	152 (100)	150 (16)	76 (16)
Acenaphthene	14.30	1.3	154 (100)	152 (57)	76 (37)
2,4-Dinitrotoluene	15.33	0.8	165 (100)	89 (66)	63 (44)
2,6-Dinitrotoluene	15.73	0.7	165 (100)	89 (58)	63 (52)
Fluorene	16.46	1.7	166 (100)	164 (21)	115 (5)
4-Chlorophenyl phenyl ether	16.67	0.9	204 (100)	141 (59)	206 (36)
Diphenylamine	17.25	11.5	169 (100)	167 (38)	141 (5)
N-Nitrosodiphenylamine[a]	17.25	—	169 (100)	167 (38)	141 (5)
4-Bromophenyl phenyl ether	18.84	0.3	248 (100)	250 (100)	141 (59)
Phenanthrene	20.75	0.3	178 (100)	176 (20)	152 (13)
Anthracene-d_{10}[b]	20.87	—	188 (100)		
Anthracene	20.94	3.0	178 (100)	176 (20)	152 (11)
Di-*n*-butyl phthalate	23.34	3.8	149 (100)	223 (7)	167 (2)
5-Nitroacenaphthene	24.02	4.4	199 (100)	152 (97)	76 (16)
Fluoranthene	24.55	0.3	202 (100)	200 (21)	101 (16)
Pyrene	25.07	0.3	202 (100)	200 (21)	101 (18)
Butyl benzyl phthalate	26.79	0.5	149 (100)	206 (42)	123 (12)
Bis(2-ethylhexyl) phthalate	27.47	2.2	149 (100)	167 (51)	279 (23)
Benzo(a)anthracene	27.68	0.5	228 (100)	226 (27)	113 (11)
Chrysene-d_{12}[c]	27.71	—	240 (100)		
Chrysene	27.76	0.3	228 (100)	226 (28)	113 (12)
Di-*n*-octyl phthalate	29.12	6.6	149 (100)	279 (17)	167 (2)
Benzo(b)fluoranthene	29.93	0.6	252 (100)	250 (22)	126 (10)
Benzo(k)fluoranthene	29.93	0.6	252 (100)	250 (22)	126 (10)
Benzo(a)pyrene	30.62	0.5	252 (100)	250 (22)	126 (10)
Perylene	30.83	1.5	252 (100)	250 (26)	126 (11)
Indeno(1,2,3cd)pyrene	33.91	2.0	276 (100)	274 (29)	136 (7)
Dibenzo(a,h)anthracene	33.94	1.3	278 (100)	276 (21)	139 (10)
Benzo(g,h,i)perylene	34.73	4.2	276 (100)	274 (20)	136 (11)
Phenol acetates					
4-Fluorophenol[c]	6.10	—	112		
Phenol	6.17	2.4	94 (100)	136 (22)	66 (14)
o-Cresol	7.35	3.7	108 (100)	79 (16)	150 (15)
m-Cresol	7.79	3.4	108 (100)	79 (18)	150 (16)
p-Cresol	7.89	3.5	108 (100)	150 (14)	79 (13)
2-Chlorophenol	8.44	3.7	128 (100)	130 (33)	170 (14)
2,4-Dimethylphenol	9.17	7.4	122 (100)	107 (58)	164 (12)
2,6-Dichlorophenol	10.86	2.0	162 (100)	164 (67)	204 (19)
4-Chloro-3-methylphenol	11.06	1.5	142 (100)	144 (34)	184 (13)
2,4-Dichlorophenol	11.28	1.7	162 (100)	164 (68)	204 (10)
2,4,6-Trichlorophenol	13.34	1.3	198 (100)	196 (99)	240 (14)
4-Nitrophenol	13.82	1.4	139 (100)	109 (99)	181 (52)
2,4,5-Trichlorophenol	14.63	1.3	198 (100)	196 (99)	240 (14)
3,4,5-Trichlorophenol	15.63	0.7	198 (100)	196 (99)	240 (14)
2,3,4-Trichlorophenol	15.94	0.6	198 (100)	196 (99)	240 (15)
2,3,5,6-Tetrachlorophenol	17.40	1.6	232 (100)	230 (75)	234 (49)
2,3,4,6-Tetrachlorophenol	17.50	2.8	232 (100)	230 (75)	234 (49)
2,3,4,5-Tetrachlorophenol	18.82	0.4	232 (100)	230 (75)	234 (49)
2,4-Dinitrophenol	19.10	NA[d]	226 (1)	43 (100)	168 (1)
4,6-Dinitro-*o*-cresol	20.87	NA	240 (1)	43 (100)	168 (1)
Pentachlorophenol	21.37	1.2	266 (100)	264 (63)	268 (69)

[a] Detected as diphenylamine. [b] Internal standard. [c] Surrogate standard. [d] Data not available.

Table 3. Recoveries of priority pollutants from organic-free water

Compound	Fortification level, μg/L	Average rec.,[a] %	CV, %
Base/neutral compounds			
Acenaphthene	20.0	101 (96–103)[b]	2.1
	10.0	102 (99–104)	1.8
	5.0	94 (80–102)	9.4
Acenaphthylene	21.4	100 (94–102)	2.5
	10.7	101 (98–102)	1.4
	5.4	94 (76–100)	9.4
Anthracene	17.2	101 (98–102)	1.3
	8.0	109 (108–111)	1.6
	4.3	86 (47–105)	27.0
Benzo(a)anthracene	30.0	97 (95–100)	2.0
	15.0	101 (99–103)	1.5
	7.5	100 (96–103)	2.0
Benzo(a)pyrene	24.3	98 (96–102)	2.3
	12.2	100 (98–102)	1.8
	6.1	97 (93–103)	3.1
Benzo(b)fluoranthene	34.4	98 (95–100)	1.8
	17.2	101 (97–105)	2.1
	8.6	99 (97–105)	2.5
Benzo(k)fluoranthene		—NA[c]—	
Benzo(g,h,i)perylene	15.2	99 (89–93)	6.5
	7.6	103 (97–108)	3.6
	3.8	95 (90–105)	5.8
Bis(2-chloroethyl) ether	39.6	98 (82–103)	6.7
	19.8	99 (97–101)	1.4
	9.9	93 (68–102)	15.9
Bis(2-chloroethoxy) methane	33.4	98 (84–102)	5.9
	16.7	100 (97–104)	2.1
	8.4	88 (63–100)	15.5
Bis(2-chloroisopropyl)ether	20.0	101 (82–111)	8.6
	10.0	99 (96–103)	2.6
	5.0	90 (66–102)	16.4
Bis(2-ethylhexyl)phthalate	40.8	98 (96–100)	1.7
	20.4	98 (89–113)	8.4
	10.2	93 (81–103)	7.6
4-Bromophenyl phenyl ether	20.8	100 (97–103)	1.9
	10.4	102 (100–104)	1.9
	5.2	100 (98–102)	1.9
Butyl benzyl phthalate	20.0	98 (95–100)	1.7
	10.0	101 (94–104)	2.7
	5.0	96 (92–104)	3.8
Camphene	30.8	84 (57–112)	22.3
	15.4	101 (100–105)	1.6
	7.7	78 (53–100)	19.7
1-Chloronaphthalene	29.9	99 (89–104)	4.4
	14.9	103 (100–106)	2.2
	7.5	89 (71–100)	12.5
2-Chloronaphthalene	20.5	100 (91–103)	3.6
	10.3	99 (96–100)	1.6
	5.1	94 (73–102)	12.5
4-Chlorophenyl phenyl ether	29.4	100 (98–102)	1.6
	14.7	101 (99–104)	1.8
	7.4	99 (89–101)	4.0
Chrysene	17.3	99 (97–100)	0.9
	8.6	100 (95–97)	2.4
	4.3	102 (100–107)	2.3
Dibenzo(a,h)anthracene	33.2	98 (88–106)	6.3
	16.6	103 (98–107)	3.0
	8.3	95 (90–104)	5.3
Di-*n*-butyl phthalate	17.9	98 (96–101)	1.8
	8.9	103 (99–117)	5.8
	4.5	109 (96–180)	25.7
Di-*n*-octyl phthalate	18.9	98 (91–104)	4.5
	9.5	72 (3–102)	64.7
	4.7	62 (4–100)	75.5
2,4-Dinitrotoluene	23.6	100 (97–102)	2.3
	11.8	101 (97–104)	2.7
	5.9	97 (93–107)	4.7
2,6-Dinitrotoluene	20.8	99 (97–105)	4.8
	10.4	100 (97–102)	2.3
	5.2	94 (87–102)	4.7
Diphenylamine	54.6	183 (98–323)	63.1
	27.3	70 (27–101)	52.9
	13.6	76 (34–101)	44.8
Diphenyl ether	32.7	98 (88–105)	1.77
Fluoranthene	19.8	99 (99–101)	0.8
	9.9	102 (99–104)	1.9
	4.9	102 (100–106)	2.2
Fluorene	15.0	101 (97–102)	1.3
	7.5	100 (97–103)	1.9
	3.7	89 (73–103)	17.3
Indeno(1,2,3-cd)pyrene		—NA—	
Indole	32.7	99 (93–102)	2.8
	16.4	101 (96–103)	2.2
	8.2	91 (78–100)	8.5
1-Methylnaphthalene	34.6	98 (87–102)	4.8
	17.3	101 (99–102)	1.4
	8.6	92 (71–100)	13.7
2-Methylnaphthalene	22.4	99 (86–103)	5.2
	11.2	101 (99–102)	1.3
	5.6	93 (70–100)	13.8
Naphthalene	26.2	98 (99–102)	6.1
	13.1	98 (96–101)	1.7
	6.5	91 (66–100)	15.6
5-Nitroacenaphthene	102.2	98 (94–102)	2.2
	51.1	100 (93–106)	3.7
	25.5	94 (89–105)	6.0
N-Nitrosodi-*n*-propylamine	28.8	96 (96–102)	7.1
	14.4	98 (79–104)	8.6
	7.2	87 (63–100)	16.3
N-Nitrosodiphenylamine		—NA—	
Perylene	50.5	97 (94–100)	1.8
	25.3	101 (96–104)	2.8
	12.6	94 (88–100)	4.2
Phenanthrene	21.2	100 (100–101)	0.5
	10.6	101 (100–103)	1.2
	5.3	100 (100–96)	2.1
Pyrene	20.0	100 (98–101)	0.9
	10.0	102 (98–104)	2.2
	5.0	102 (98–104)	2.1
Phenolic compounds			
4-Chloro-3-methylphenol	19.8	99 (93–104)	3.2
	9.9	100 (97–103)	1.8
	5.0	88 (72–98)	5.2
2-Chlorophenol	36.0	99 (85–104)	5.9
	18.0	99 (97–102)	1.7
	9.0	86 (63–100)	16.0
m-Cresol	31.0	97 (84–102)	6.0
	15.5	103 (99–106)	2.6
	7.8	83 (60–100)	17.5
o-Cresol	29.8	96 (82–103)	7.6
	14.9	101 (96–105)	3.3
	7.5	80 (53–100)	20.4
p-Cresol	33.1	97 (84–103)	6.0
	16.5	99 (94–103)	3.2
	8.3	86 (61–100)	17.5
2,4-Dichlorophenol	20.0	101 (93–104)	3.2
	10.0	101 (98–103)	1.9
	5.0	90 (72–100)	12.2
2,6-Dichlorophenol	22.0	99 (90–103)	4.0
	11.0	99 (97–102)	1.7
	5.5	87 (67–100)	14.0
2,4-Dimethylphenol	59.2	96 (83–106)	8.6

Table 3. Continued

Compound	Fortification level, μg/L	Average rec.,[a] %	CV, %
Base/neutral compounds			
	29.6	99 (93–103)	3.7
	14.8	76 (51–100)	21.9
2,4-Dinitrophenol	100	—Nil—	
4,6-Dinitro-*o*-cresol	100	—Nil—	
4-Nitrophenol	39.6	100 (97–105)	2.6
	19.8	100 (96–106)	3.2
	9.9	93 (85–100)	5.0
Pentachlorophenol	28.6	99 (92–106)	4.5
	14.3	101 (97–106)	2.4
	7.2	92 (83–100)	6.2
Phenol	22.6	97 (82–100)	6.7
	11.3	112 (97–101)	1.6
	5.7	86 (63–100)	16.3
2,3,4,5-Tetrachlorophenol	19.8	98 (94–102)	2.6
	9.9	98 (78–105)	8.9
	5.0	96 (92–100)	2.5
2,3,4,6-Tetrachlorophenol	18.8	91 (71–103)	14.6
	9.4	119 (100–136)	10.5
	4.7	113 (96–145)	17.7
2,3,5,6-Tetrachlorophenol	20.4	112 (97–135)	13.7
	10.2	89 (75–104)	13.8
	5.4	92 (79–103)	10.4
2,3,4-Trichlorophenol	20.4	100 (98–102)	1.4
	10.2	102 (99–106)	2.5
	5.1	94 (90–100)	4.0
2,4,5-Trichlorophenol	33.8	96 (96–97)	2.6
	16.9	100 (99–103)	3.2
	8.5	94 (87–100)	5.0
2,4,6-Trichlorophenol	20.4	99 (94–102)	3.0
	10.2	101 (97–102)	2.1
	5.1	92 (78–96)	9.4
3,4,5-Trichlorophenol	19.8	101 (98–104)	2.3
	9.9	102 (98–105)	2.2
	5.0	94 (88–100)	4.9

[a] Average relative recovery for 8 recovery measurements.
[b] The range of 8 recovery measurements is given in parentheses.
[c] Data not available.

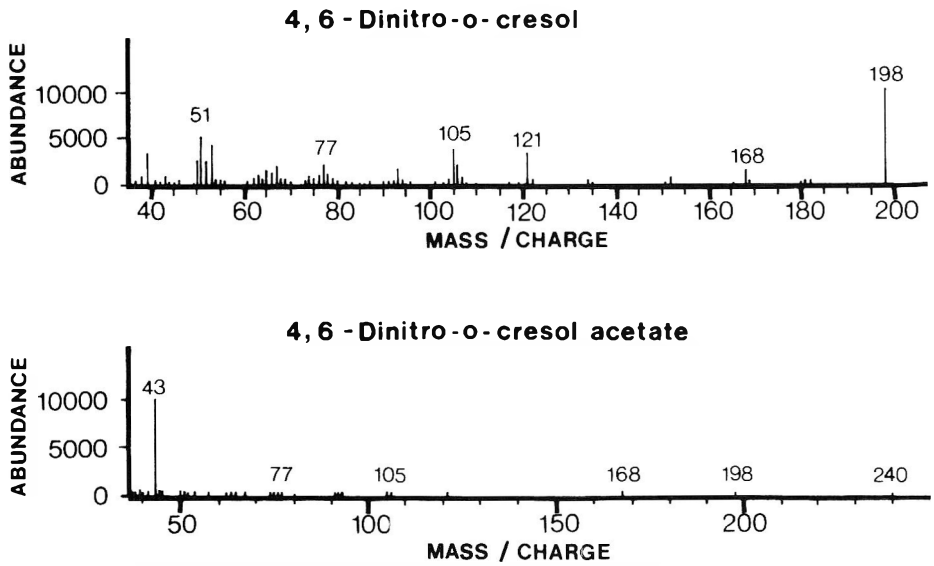

Figure 1. Mass spectra of 4,6-dinitro-*o*-cresol and 4,6-dinitro-*o*-cresol acetate.

Table 4. Recoveries of selected organic compounds from water samples collected from a landfill site

Compound	Fortification level, μg/L	Rec. range,[a] %
2-Chlorophenol	40.0	65–80
2,3,4,6-Tetrachlorophenol	50.0	92–148
4-Nitrophenol	140.0	56–99
Bis(2-chloroethoxy)methane	40.0	73–98
Bis(2-ethylhexyl)phthalate	50.0	36–100
5-Nitroacenaphthene	100.0	65–100
Perylene	560.0	48–90
Benzo(g,h,i)perylene	25.0	16–72

[a] Based on 5 samples.

chlorophenols, chloroguaiacols, chlorosyringol, chlorovanillin, chlorocatechols), and other matrixes (e.g., soil, sediment) is under investigation in this laboratory.

The method described is rapid and cost-effective. The chromatography and sensitivities of the acetylated phenols vs underivatized phenols are enhanced as a result of considerable reduction in peak tailing and improvement in peak symmetry/integration. The accuracy and precision of the measurements are enhanced compared to previous methods.

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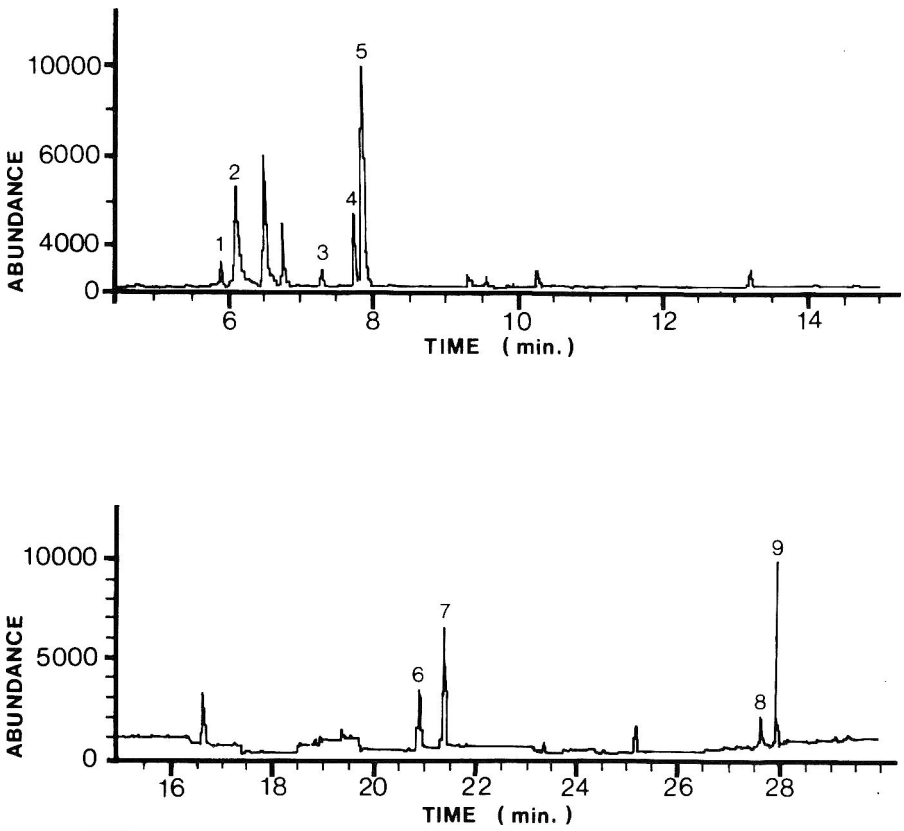

Figure 2. Total ion chromatogram of wastewater sample. Experimental conditions given in method.

Target compounds identified: 1, 4-fluorophenol (surrogate standard); 2, phenol; 3, *o*-cresol; 4, *m*-cresol; 5, *p*-cresol; 6, anthracene-d_{10} (internal standard); 7, pentachlorophenol; 8, chrysene-d_{12} (surrogate standard); and 9, bis-2(ethylhexyl) phthalate. Other peaks were not identified.

Ontario, Canada, and C. Chan, and J. Martin of Mann Testing Laboratories Ltd, Mississauga, Ontario, Canada, for valuable discussions during this study.

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EXTRANEOUS MATERIALS AND FILTH IN FOODS

Chemical Test for Mammalian Feces in Grain Products: Collaborative Study

HARRIET R. GERBER
Food and Drug Administration, Division of Microbiology, Washington, DC 20204

Collaborators: J. R. Bryce; M. Chaput; V. B. Deblois; G. R. Dzidowski; D. H. Floyd; M.-A. Gardiner; R. Klein; D. Mayers; J. A. McDonnell; J. C. Romer; R. L. Trauba; F. D. McClure and L. K. Bullock (*Statistical Consultants*)

A collaborative study was conducted to validate the use of the AOAC alkaline phosphatase method for mammalian feces in corn meal, 44.B01–44.B06, for 7 additional products: brown rice cream, oat bran, grits, semolina, pasta flour, farina, and barley plus (a mixture of barley, oat bran, and brown rice). The proposed method determines the presence of alkaline phosphatase, an enzyme contained in mammalian feces, by using phenolphthalein diphosphate as the enzyme substrate in a test agar medium. Fecal matter is separated from the grain products by specific gravity differences in 1% test agar. As the product is distributed on liquid test agar, fecal fragments float while the grain products sink. The alkaline phosphatase cleaves phosphate radicals from phenolphthalein diphosphate, generating free phenolphthalein, which produces a pink to red-purple color around the fecal particles in the previously colorless medium. Collaborators' recovery averages ranged from 21.7 particles (72.3%) for oat bran to 25.3 particles (84.3%) for semolina at the 30 particle spike level. Overall average background was 0.4 positive reactions per food type. The collaborators reported that the method was quick, simple, and easy to use. The method has been approved interim official first action for all 7 grain products.

The official AOAC method for determining the presence of rodent excreta in grain products (1) uses sedimentation in chloroform and carbon tetrachloride to separate and recover fecal particles. Recovery of small fecal particles is poor because the particles tend to float throughout the solvents instead of settling to the bottom (2). Also, small fecal particles that are recovered usually do not contain hair or have a mucous coating and therefore can seldom be positively identified as feces.

A method was developed to replace the official AOAC method for identifying rodent excreta in corn meal (3). The method separates the product and feces by differences in specific gravity in a 1% test agar medium and determines fecal particles by showing the presence of alkaline phosphatase, a fecal enzyme. Borate buffer maintains the test medium at pH 9.5. Both phosphate radicals must be cleaved from the substrate before any color will appear, and this occurs by the specific action of the phosphatase. The 1% gelled agar retards diffusion of color, thereby concentrating the color around the small particles and facilitating the identification.

The present collaborative study applies the new corn meal method to an additional 7 products. This enzymatic analysis can identify particles as small as 250 μm, a very low level for feces. The method, which is relatively simple and rapid to perform (1.5 h total analytical time), uses less hazardous chemicals than does the official method (1).

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This report was presented at the 101st AOAC Annual International Meeting, September 14–17, 1987, at San Francisco, CA.
The recommendation has been approved interim official first action by the General Referee, the Committee on Microbiology and Extraneous Materials, and the Chairman of the Official Methods Board. The method will be submitted for adoption at the 103rd AOAC Annual International Meeting, September 25–28, 1989, at St. Louis, MO. Association actions will be published in "Changes in Official Methods of Analysis" (1990) *J. Assoc. Off. Anal. Chem.* **73**, January/February issue.

Collaborative Study

The grain products included in this study were brown rice cream, oat bran, grits, semolina, farina, and barley plus (a mixture of barley, oat bran, and brown rice). These products, obtained from commercial sources, were analyzed for background positives (i.e., spots seen in the food before addition of the spike). For each of the 7 products, a minimum of three 10 g portions were tested and showed a background of 0–2 positive spots per 10 g portion.

Each collaborator was sent 1 practice and 4 test samples for each of the 7 grain products, totaling 7 practice and 28 test samples. The practice test sample consisted of 10 g of product to which 30 fecal particles had been added. The 4 test samples of each product consisted of two 10 g portions with no fecal particles added (the 0 particle spike level) and two 10 g portions containing 30 fecal particles (the 30 particle spike level). Collaborators were also sent substrate indicator phenolphthalein diphosphate, mouse fecal particles for positive controls, the proposed method, instructions, recording forms, and a comments sheet. Expected minimum recoveries for the practice samples were given, and collaborators were requested to call if these minimum results were not obtained.

Mammalian Feces in Grain Products
Alkaline Phosphatase Detection Method
Interim First Action

(Applicable to brown rice cream, oat bran, grits, semolina, pasta flour, farina, and barley plus)

Method Performance:

Brown rice cream

$s_r = 4.3$; $s_R = 5.3$; $RSD_r = 17.8\%$; $RSD_R = 22.0\%$

Oat bran

$s_r = 5.5$; $s_R = 7.8$; $RSD_r = 25.2\%$; $RSD_R = 36.0\%$

Grits

$s_r = 3.4$; $s_R = 5.6$; $RSD_r = 15.1\%$; $RSD_R = 25.2\%$

Semolina

$s_r = 3.6$; $s_R = 4.7$; $RSD_r = 14.4\%$; $RSD_R = 18.7\%$

Pasta flour

$s_r = 5.5$; $s_R = 5.5$; $RSD_r = 22.0\%$; $RSD_R = 22.0\%$

Farina

$s_r = 2.3$; $s_R = 4.4$; $RSD_r = 9.2\%$; $RSD_R = 18.1\%$

Barley plus

$s_r = 4.1$; $s_R = 6.1$; $RSD_r = 17.5\%$; $RSD_R = 26.0\%$

A. Principle

Intestinal tract of most mammals contains alkaline phosphatase enzyme. Enzyme at test pH and temperature splits phosphate radicals from substrate/pH indicator phenolphthalein diphosphate to produce light pink to red-purple color from free phenolphthalein.

B. Apparatus

(a) *Hot water bath.*—Maintained at 42 ± 1°.

(b) *Hot plate stirrer and stirring bar.*—See **44.002(n).** 41 mm ovoid stirring bar is recommended.

(c) *Petri dishes.*—Plastic, disposable, or glass. 150 × 20 mm or 150 × 15 mm.

(d) *Weighing boats.*—8.1 × 8.1 × 1.9 cm, 100 mL capacity, or approximate equivalent size.

C. Reagents

(a) *Magnesium chloride solution.*—Dissolve 0.203 g $MgCl_2.6H_2O$ in water and dilute to 500 mL with water. Solution has indefinite shelf life.

(b) *Stock test reagent.*—Dissolve 19.0 g borax ($Na_2B_4O_7$. 10 H_2O) and 6.28 g anhydrous Na_2CO_3 in 1 L H_2O with stirring. Add 0.94 g phenolphthalein diphosphate (Sigma P 9875 or equivalent), and stir while adding 2 mL $MgCl_2$ solution. Preparation is stable ca 4 months at room temperature. Solution should be colorless and ca pH 9.5. Discard if not colorless. Degraded phenolphthalein diphosphate produces pink color in reagent. Store phenolphthalein diphosphate in desiccator below 0°.

(c) *Liquid test agar.*—Prepare fresh daily and maintain at 42°; 150 mL per plate is needed. Measure equal volume of stock test reagent, **(b)** (half of total test agar volume needed), and of water into separate beakers of appropriate size. Beaker for water must be large enough to accommodate 2 times the volume of water. Reserve stock test reagent. Place beaker of water on hot plate stirrer, add stirring bar, and with rapid stirring, add sufficient agar to water to yield 2% agar solution (1.5 g agar/75 mL H_2O). Continue stirring, and heat to boil (watch for foam-over). Place cover glass on beaker 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 solution. Stir rapidly over heat ca 1 min.

D. Determination

Weight 10 g from each well-mixed subsample of product into weighing boat. For each 10 g sample, use number of plates appropriate for product/feces visibility per product as follows: 2 plates each for grits and semolina flour and 3 plates each for oat bran, brown rice cream, pasta flour, farina, and barley plus. Cool boiled test agar by placing beaker in larger beaker of cold water. Continually *stir* test agar and *maintain* temperature check until solution is 42°. Pour test agar into petri dish, ca 150 mL per dish. Immediately distribute monolayer of product onto surface of test agar by gently tapping weighing boat; hold boat so that product flows over one side, not from a corner, tilting and moving boat above agar surface as product flows. Let product become wet with test agar before adding another layer. Distribute sample evenly among appropriate number of petri dishes. Continue in this manner until entire 10 g sample has been added. Distribution time should be ca 1 min/10 g sample. Separation of product and fecal particles is best while test agar is hot. Multiple samples can be added to respective dishes one at a time, or a little of each sample to its respective dishes sequentially, until all of each sample has been distributed. If clumping is observed while sample is added, gently swirl plate before continuing. Swirl only when clumps form.

Let test agar gel with lids off petri dishes (requires ≥20 min). Agar is gelled when no agar flows when dish is slightly tipped. (*Note:* Take care not to disturb dispersed material in liquid test agar. If particles are moved, color concentration around particles will be diffused and positive spots will be missed.) An electric fan, blowing gently on petri dishes, may be used to hasten cooling and gelling. When agar is firm, check for pink to red-purple spots, viewing plate against white background. Color of positive spots should be same as that seen in positive control plate. (Be careful not to count spots that are reddish brown or that have another added color. Extra colors are dyes that leach from grain products themselves.) Examine both top and bottom surfaces of each plate so that line of sight is perpendicular to plate while plate is held at 45° angle to white background on table, with light source directed onto plate from above. View plates from side as well as top and bottom. Mark positive spots on lid of dish, using grease pencil, and mark lid and bottom together, using waterproof marker, so that lid can always be placed in same position.

Incubate petri dishes 10 min in 42° water bath. Submerge plates in bath just enough to cover agar level in dishes. When several dishes are incubated at one time, place plates in water in pairs, staggering times so that reading delays are avoided and small, rapidly diffusing pink spots are not missed. Remove plates from water bath after 10 min. Wipe inside of 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 plate is read. Mark spots as before. Replace lid and repeat 10 min incubation 2 times, marking additional pink spots on petri dish lid after each incubation period. Tally and record number of spots as fecal particles per 10 g sample. Spots that appear and then are not seen on subsequent checks and spots that are seen on bottom of petri dish with product are to be counted in tally.

E. Positive Control for Feces and Test Agar Medium

Scatter some ground, known rodent feces on petri dish of liquid test agar in place of food and continue with method. One control plate is needed for each batch of test agar prepared.

F. Response

Amount, intensity, and range of color (light pink to red-purple) observed will vary depending on size of fecal particles, species, and diet of animal. Particles as small as 250 μm can be identified.

Ref.: JAOAC **72,** September/October issue (1989).

Results and Discussion

The results of the collaborative study are presented in Tables 1 and 2. Results submitted by Collaborator E have not been included in the statistical analysis because reddish brown spots were mistakenly counted as positive. A statement cautioning analysts to count only spots similar to control spots has been added to the method. This caution also applies to corn meal.

The average number of positive particles reported by collaborators for each product at the 0 spike level ranged from 0 for barley plus to 1.6 particles for oat bran (Table 1). Precollaborative studies also showed low background counts for these products. Specifically, brown rice cream, semolina,

Table 1. Collaborative results for recovery of fecal particles added to grain products, 0 particle spike level

	Brown rice cream		Oat bran		Grits		Semolina		Pasta flour		Farina		Barley plus	
Coll.	1	2	1	2	1	2	1	2	1	2	1	2	1	2
A	0	0	3	0	0	0	0	0	0	0	0	0	0	0
B	0	0	0	0	0	0	0	2	0	0	0	0	0	0
C	0	0	0	3	0	0	0	0	0	0	0	0	0	0
D	0	0	12	0	0	0	0	0	0	0	0	0	0	0
E[a]	14	3	6	4	3	0	15	47	0	7	15	7	24	5
F	1	11	5	3	0	0	0	0	0	0	1	3	0	0
G	0	0	2	4	0	0	0	0	1	0	1	2	0	0
H	0	0	1	0	0	0	0	0	0	0	0	0	0	0
I	1	0	0	0	0	0	0	0	0	0	1	0	0	0
J	0	0	0	0	2	0	1	0	0	0	0	0	0	0
K	1	0	0	0	0	0	0	0	0	0	0	0	0	0
$\bar{X}$	0.7		1.6		0.1		0.1		0.05		0.4		0	
s_r	2.3		2.8		0.4		0.3		0.2		0.5		0	
s_R	2.5		2.8		0.4		0.3		0.5		0.8		0	
RSD_r, %	322.6		177.2		447.2		333.3		447.2		136.9		0	
RSD_R, %	351.7		177.3		447.2		333.3		447.2		208.3		0	

[a] Technical outliers were not included in the statistical analysis (see text).

Table 2. Collaborative results for recovery of fecal particles added to grain products, 30 particle spike level

	Brown rice cream		Oat bran		Grits		Semol na		Pasta flour		Farina		Barley plus	
Coll.	1	2	1	2	1	2	1	2	1	2	1	2	1	2
A	25	19	18	21	16	18	19	28	20	22	19	23	17	21
B	18	32	10	28	25	19	26	28	22	23	29	29	31	25
C	26	27	25	22	36	26	26	27	26	23	20	26	21	18
D	17	18	19	26	26	17	19	22	13	24	24	27	20	23
E[a]	39	53	29	46	30	28	29	39	34	39	35	36	46	38
F	33	30	30	33	27	29	29	27	30	28	28	26	37	25
G	30	29	23	23	24	24	31	28	29	29	31	32	28	31
H	23	19	12	0[a]	27	27	15	27	14	26	19	21	19	13
I	22	17	18	6	17	16	18	20	28	29	16	20	23	16
J	28	19	28	24	15	17	30	27	25	27	24	28	21	21
K	24	24	23	24	22	21	29	30	28	26	26	25	26	31
$\bar{X}$	24		21.7		22.4		25.3		25.1		24.6		23.3	
$\bar{X}$, %	80.0		72.3		74.7		84.3		83.7		82.0		77.7	
s_r	4.3		5.5		3.4		3.6		5.5		2.3		4.1	
s_R	5.3		7.8		5.6		4.7		5.5		4.4		6.1	
RSD_r, %	17.8		25.2		15.1		14.4		22.0		9.2		17.5	
RSD_R, %	22.0		36.0		25.2		18.7		22.0		18.1		26.0	

[a] Technical outliers by laboratory not included in statistical analysis (see text).

pasta flour, and barley plus had 0 background counts; the others averaged <1 particle per test portion (farina, 0.2; oat bran, 0.8; grits, 0.25).

The 30 particle spike level yielded average recoveries ranging from 21.7 particles (72.3%) for oat bran to 25.3 particles (84.3%) for semolina (Table 2). The overall average for the 7 products was 23.7 particles (79%). Precollaborative study recoveries by the Associate Referee were higher than those of the collaborators, with an overall average of 26.7 particles (89%). Average recoveries of particles for individual products were oat bran, 23.4 (78%); brown rice cream, 29.0 (96.7%); barley plus, 26.2 (87.3%); pasta flour, 28.0 (93.3%); farina, 28.6 (95.3%); grits, 26.0 (86.7%); and semolina, 25.8 (86%).

As previously noted (2), not all spike particles give a positive response. Feces of mice fed sunflower seeds were used in this study because a sunflower seed diet produced a high proportion of positive particles similar to that found in wild mouse feces (unpublished data). To determine the percent positive for the spike used in this study, 19 packets of fecal particles containing 30 particles per packet were tested on gelled test agar. An average of 29.0 (96.6%) positive particles was found.

Collaborators commented that the pasta flour and sometimes the brown rice cream formed clumps. High humidity seemed to cause more clumping. Even with clumps, however, the collaborators' recoveries were good. Because high humidity and heat cause phenolphthalein diphosphate to degrade faster than normal, the reagent powder should be purchased in small quantities to avoid excessive holding and handling.

A few collaborators saw a yellow hue around some particles in the barley plus and farina. No positives were seen in

the yellow area and there were concerns that the yellow color might mask the color of a positive spot; however, these collaborators recovered a high number of spike particles, indicating that there was no problem.

As noted earlier, one collaborator (E) mistakenly counted reddish brown spots, and the counts for both 0 and 30 particle spike levels were therefore consistently high. The reddish brown spots are due to color leaching from food into the agar. A few other collaborators saw these spots in the barley plus and farina, but did not count them. Extraneous spots may also be seen during analysis of corn meal. Data from collaborator E were not included in the statistical analysis.

Collaborator H did not check the temperature of the test agar and believed that in one sample the oat bran may have been added before the test agar had cooled to 42°C. No positives were seen in this sample, probably because the heat of the test agar deactivated the alkaline phosphatase. Data for this sample were not included in the statistical analysis.

Collaborators found that having a positive control plate for each run served as a reference to help distinguish positive spots from miscellaneous spots on test plates. One collaborator noted that care must be taken in counting spots when 2 particles are close together because overlapping spots could be mistaken for 1 spot. Another collaborator observed movement of the agar in dishes as they were removed from the water bath and was concerned about losing sight of some positive spots as a result of excessive dispersion of color in the agar. This situation can usually be avoided by letting test agar gel completely before placing petri dishes into the water bath. A note has been added to the method that an electric fan, blowing gently on the dishes, can be used to hasten gelling of the agars; this also applies to analysis of corn meal.

All collaborators liked the method, agreed that it was simple and easy to perform, and appreciated the elimination of hazardous solvents (i.e., carbon tetrachloride and chloroform), which are used in the present method to determine feces in grain products (1).

Recommendation

The proposed method gives high recoveries of fecal particles, eliminates the use of toxic chemicals, and is rapid to perform. It is recommended that the alkaline phosphatase method, a chemical test for mammalian feces in grain products, be adopted official first action for the following grain products: brown rice cream, oat bran, grits, semolina, pasta flour, farina, and barley plus.

It is further recommended that the method for corn meal, **44.B01–44.B06,** be combined with the method for additional grain products described in the present study. To incorporate the cautionary and clarifying statements developed here, the statement "1 plate each for corn meal," should be added immediately after the words "the number of plates appropriate to product as follows" in the Determination section. This method will then apply to all 8 grain products.

It is also recommended that the method be included as an additional method under Filth in Grain Products as the Alkaline Phosphatase Test for Mammalian Feces, applicable to corn meal, brown rice cream, oat bran, grits, semolina, pasta flour, farina, and barley plus. The method does not replace **44.049** for rodent excreta because it is not applicable to whole wheat flour and has not been tested with rye meal; **44.049** includes steps preliminary to the extraction of light filth in **44.050.**

The cautionary and clarifying statements developed in the present study do apply to the method for corn meal, **44.B01–44.B06.**

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(1) *Official Methods of Analysis* (1984) 14th Ed., AOAC, Arlington, VA, sec. **44.049**

(2) Gerber, H. R. (1986) *J. Assoc. Off. Anal. Chem.* **69,** 496–498

(3) *Official Methods of Analysis* (1984) 14th Ed., AOAC, Arlington, VA, secs **44.B01–44.B06**

FEEDS

Generic Combustion Method for Determination of Crude Protein in Feeds: Collaborative Study

ROSE A. SWEENEY[1]
University of Missouri, Experiment Station Chemical Laboratories, Agriculture Building, Columbia, MO 65211

Collaborators: O. Abara; M. Anson; L. Cartwright; R.D. Cathey; S. Cook; C. Kamper; P.F. Kane; A. Orcutt; L. Peterson; R.L. Schwarberg; P. Skelton; D.F. Tate; G.I. Willkens

Nine laboratories participated in a collaborative study on determination of crude protein in animal feeds to compare a generically described combustion method with the AOAC mercury catalyst Kjeldahl method (7.015). The combustion method was written in general terms of method principle, apparatus specifications, and performance requirements. The sample set comprised closely matched pairs of feed ingredients and mixed products ranging from 10 to 90% protein. Ten pairs ground to 0.5 mm were the focus of the study; 4 pairs were ground to 1.0 mm for comparison. Nicotinic acid and lysine monohydrochloride were included as standards. Collaborators were instructed to report their results for performance checks using materials supplied. Only one laboratory failed to meet the proposed limits. Seven laboratories used the LECO Model FP-228 analyzer and 2 used the LECO CHN 600 analyzer. For the 0.5 mm pairs, repeatability standard deviations (s_r) ranged from 0.09 to 0.58 for the Kjeldahl method and from 0.14 to 0.33 for the combustion method, with a pooled s_r value of 0.28 and relative standard deviation (RSD_r) of 0.59%. Reproducibility standard deviations (s_R) ranged from 0.23 to 0.86 (Kjeldahl) and from 0.30 to 0.61 (combustion), with a pooled s_R value of 0.52 and RSD_R of 1.10%. Grand means for the samples ground to 0.5 mm were 47.65% protein by the combustion method and 47.41% protein by the Kjeldahl method. For samples ground to 1.0 mm, corresponding values were 31.82 and 31.50% protein. The generic combustion method has been approved interim official first action.

New designs in instrumentation have shown combustion methods to be promising and practical alternatives to manual Kjeldahl methods for determination of crude protein in feeds. Measuring nitrogen by combustion appears advantageous over wet chemical methods because of the speed (3 min per determination) and freedom from harsh chemicals.

In a recent report, Sweeney and Rexroad (1) showed that the LECO FP-228 combustion analyzer method compared favorably with the AOAC copper catalyst Kjeldahl method for crude protein determination **(7.033–7.037)** (2). The present report describes a collaborative study to compare a combustion method written in generic terms with the AOAC mercury catalyst Kjeldahl method **(7.015)** (2).

Collaborative Study

The combustion method was written in generic style and included performance criteria to avoid requiring a particular manufacturer's instrument. Key features of the instrumentation are the focus for the description. Performance guidelines direct application of the method to determination of crude protein in animal feeds. These guidelines center around ensuring accurate measurement and determining the appropriate fineness of grind for nonhomogeneous samples.

Nine laboratories participated in the study. Capability to carry out both methods was a requirement for participation. Of the 9 participants, 7 used the LECO Model FP-228 combustion analyzer and 2 used the LECO CHN 600 combustion analyzer. These instruments are probably the most practical available because they can handle a ≥ 100 mg sample, which is especially critical in analysis of nonhomogeneous materials. Sample grind influences the precision of the combustion method and is addressed in this study.

The sample set comprised 30 samples as 2 groups of closely matched pairs. One group, the focus of the study, consisted of 10 pairs of materials ground to pass a 0.5 mm screen. The other group was 4 pairs ground to pass a 1.0 mm screen. Two standard materials, nicotinic acid and lysine·HCl, were also included. In addition to the 30 samples, 3 materials were provided for performance tests on the combustion method.

Protein (Crude) in Animal Feed—Combustion Method Interim First Action

(Applicable to solid feeds containing 0.2–20% N)

Method Performance:

s_r = 0.28; s_R = 0.52; RSD_r = 0.59%; RSD_R = 1.10%

A. Principle

Nitrogen freed by combustion at high temperature in pure oxygen is measured by thermal conductivity detection and converted to equivalent protein by appropriate numerical factor.

B. Apparatus

Any instrument or device designed to measure nitrogen by combustion may be used, which is equipped to provide following conditions. *Caution:* Follow manufacturer's recommendation for safe operation of instrument.

(a) *Furnace.*—To maintain minimum operating temperature of 950° for pyrolysis of sample in pure (99.9%) oxygen. Some systems may require higher temperature.

(b) *Isolation system.*—To isolate liberated nitrogen gas from other combustion products for subsequent measurement by thermal conductivity detector. Device for converting NO_x products to N_2 or measuring N as NO_2 may be required and included in the design.

(c) *Detection system.*—To interpret detector response as % nitrogen w/w. May include features such as calibration on standard material, blank determination, and barometric pressure compensation. Any required calibration must be passed on theoretical % nitrogen in pure primary standard organic material such as EDTA.

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The recommendation has been approved interim official first action by the General Referee, the Committee on Feeds, Fertilizers, and Related Materials, and the Chairman of the Official Methods Board. The method will be submitted for adoption at the 103rd AOAC Annual International Meeting, September 25–28, 1989, at St. Louis, MO. Association actions will be published in "Changes in Official Methods of Analysis" (1990) *J. Assoc. Off. Anal. Chem.* **73**, January/February issue.
Contribution from the Missouri Agricultural Experiment Station. Journal Series Number 10848.
[1] Current address: Analytical Bio-Chemistry Laboratories, Inc., PO Box 1097, Columbia, MO 65205.

Table 1. Description of collaborative samples for study of combustion method for determination of crude protein in feeds

Closely matched pair	Sample No.	Estd % protein	Grind, mm	Description
1	1	88	0.5	blood meal
	22	88		
2	3	46	0.5	soybean oil meal
	28	44		
3	7	85	0.5	beef finisher, high NPN
	12	81		
4	8	16	0.5	swine grower
	5	15		
5	10	52	0.5	meat & bonemeal
	17	48		
6	16	66	0.5	poultry diet
	26	59		
7	19	11	0.5	swine diet
	29	10		
8	23	21	0.5	corn/soybean mix
	25	20		
9	24	13	0.5	swine finish
	18	13		
10	30	89	0.5	feather meal
	15	81		
11	2	21	1.0	corn/soybean mix
	20	20		
12	4	11	1.0	swine diet
	21	10		
13	9	84	1.0	beef finisher, high NPN
	11	80		
14	27	13	1.0	swine finish
	13	13		
—	6	71	—	nicotinic acid standard
	14	96	—	lysine·HCl standard

C. Performance Requirements

System equipped as in **(B)** must meet or exceed following minimum performance specifications.

(*1*) System must be capable of measuring nitrogen in feed materials containing 0.2–20% nitrogen.

(*2*) Accuracy of system is demonstrated by making 10 successive determinations of nitrogen in nicotinic acid and 10 successive determinations in lysine·HCl. Means of determinations must be within ±0.15 of the respective theoretical values, with standard deviations ≤ 0.15. Standard tryptophan may be substituted for lysine·HCl.

(*3*) Suitable fineness of grind is that which gives relative standard deviation (RSD) ≤ 2.0% for 10 successive determinations of nitrogen in mixture of corn grain and soybeans (2 + 1) that has been ground for analysis. RSD, % = (SD/mean % N) × 100.

Fineness (ca 0.5 mm) required to achieve this precision must be used for all mixed feeds and other nonhomogeneous materials.

D. Calculation

Crude protein, % = % N × 6.25, or % N × 5.70 in case of wheat grains.

Ref.: JAOAC **72,** September/October issue (1989).

Results and Discussion

The 30 study samples are identified in Table 1. In general, closely matched pairs were obtained by securing different lots of the same product. Collaborators were instructed to assay each sample once by the combustion method and once by AOAC mercury catalyst Kjeldahl method **7.015** (2). The results from the 9 laboratories are shown in Table 2.

Collaborators were also instructed to do the performance tests for the combustion method using the materials provided and to report their results. These tests for method accuracy and for establishing sample grind are in the method section on performance requirements. A mixture of corn and soybeans (2 + 1) ground to 0.5 mm was used to establish sample grind. The results from these tests were requested to help establish whether the tolerances given were reasonable. The results (Table 3) show that the minimum accuracy requirements (average for 10 determinations each—nicotinic acid, 11.38 ± 0.2%, SD 0.25 and lysine·HCl, 15.34 ± 0.3%, SD 0.35) were easily met by most laboratories which suggests that the tolerances should be made tighter. The sample grind test (RSD ≤ 2.0% for 10 determinations) using a material ground to 0.5 mm was met by all laboratories except laboratory 5, so it is judged to be reasonable.

Several collaborators commented that running 10 lysine·HCl samples in succession on the model FP-228 instrument for the accuracy test tended to clog the iron chip filter so it had to be replaced often during the test. The results are quite good in spite of this annoyance. Another material could be used in place of lysine·HCl to avoid this idiosyncrasy.

Two-sample X-Y plots of the 14 sample pairs were prepared according to Youden (ref. 3, pp. 10–11) and examined for gross outliers for both methods.

Of the 14 possible pairs for the Kjeldahl method, 9 values reported by laboratory 3 were gross outliers. These were predominantly in the high (++) quadrant. Laboratory 5 was consistently in the low (−−) quadrant for both methods. Further ranking of the laboratories according to the Cochran method, explained in Steiner (ref. 3, p. 74), confirmed the consistently low results reported by laboratory 5 for both methods.

In addition, an analysis of variance of the X-Y differences of the ten 0.5 mm pairs, shows a lab. × method interaction when laboratories 3 and 5 are included in the test, but does not when they are removed. Grand means of the X-Y differences were 2.46 and 2.65 for 9 laboratories vs 7 laboratories, respectively, for the Kjeldahl method, and 2.62 and 2.62, respectively, for the combustion method. Based on these tests, the results from laboratories 3 and 5 for both methods were excluded from further statistical treatment of the results. The difficulties were predominately associated with the Kjeldahl method.

The remaining results were processed for precision estimates according to Youden's scheme of closely matched pairs (ref. 3, pp. 22–23). Table 4 shows the 10 pairs representing the 0.5 mm ground materials arranged in order of increasing % protein. Standard deviations for the combustion method were lower than standard deviations for the Kjeldahl method for high protein materials (pairs 1, 3, 6, and 10) and higher than Kjeldahl results for low protein materials (pairs 4, 7, and 8). The combustion method does not show the same effect of increasing standard deviation with higher protein level as does the Kjeldahl method. Pooled estimates of standard deviations for the combustion method were calculated. Standard deviations for repeatability (s_r) and reproducibility (s_R) were 0.23 and 0.52, respectively; relative standard deviations (RSD_r and RSD_R) were 0.59 and 1.10%, respectively.

For the 4 pairs representing the 1.0 mm ground materials, the standard deviations for the combustion method were generally higher than those of the Kjeldahl method (Table 4). In some cases, the bias component (s_L) could not be estimated and is shown as a < 0 value. This has the effect of

Table 2. Collaborative results for determination of crude protein (%) in feeds by AOAC Kjeldahl method 7.015 and a generic combustion method

	1		2		3[a]		4		5[a]		6		7		8		9	
Sample No.	Kjel.	Comb.	Kjel.	Comb.	Kjel.	Comb.	Kjel.	Comb.	Kjel.	Comb.	Kjel.	Comb.	Kjel.	Comb.	Kjel.	Comb.	Kjel.	Comb.
1	88.69	87.64	87.70	88.81	89.12	88.58	87.24	88.47	86.47	86.43	88.90	87.78	88.20	88.06	88.24	88.50	89.35	88.24
2	20.63	20.14	20.35	19.25	20.43	20.48	19.81	21.54	19.92	19.90	20.40	20.31	20.07	20.00	20.65	20.43	20.68	20.64
3	47.44	46.45	46.32	46.69	49.17	46.90	46.32	47.13	45.79	45.83	46.74	46.86	46.42	46.25	46.84	47.11	46.81	47.09
4	11.19	10.91	10.91	11.38	12.88	10.95	10.72	11.66	10.89	10.98	11.45	12.31	10.97	11.38	11.26	11.32	11.16	11.35
5	15.63	15.26	15.27	15.12	16.75	15.73	15.18	15.63	14.83	14.90	15.38	15.94	15.25	15.56	15.60	15.89	15.35	15.72
6	71.63	70.38	71.21	71.21	63.58	71.69	68.29	71.36	18.31	70.42	66.48	71.64	55.71	71.44	71.57	71.50	71.68	71.28
7	85.50	85.38	83.79	85.56	84.43	85.26	83.83	85.20	83.41	83.58	84.66	84.44	84.72	84.88	84.60	84.96	85.38	84.73
8	15.88	15.89	15.44	15.69	15.78	15.83	15.37	15.08	15.17	15.02	15.60	16.43	15.69	15.75	16.05	15.98	15.80	16.51
9	83.50	84.16	84.15	84.50	87.65	82.26	84.42	84.39	81.74	81.71	83.16	82.85	84.88	86.25	84.32	84.59	82.78	83.05
10	52.00	52.05	52.14	51.94	53.31	52.18	51.19	51.73	50.80	50.84	50.97	52.18	51.59	52.25	52.20	52.44	51.65	52.19
11	80.25	80.86	80.55	81.06	89.65	79.43	79.87	80.08	79.27	79.01	78.99	81.16	80.43	81.00	81.13	81.16	81.86	80.93
12	81.69	81.71	81.01	82.44	81.28	82.15	80.81	81.76	79.85	79.74	80.00	80.69	81.18	81.44	81.66	81.71	82.17	81.94
13	13.38	13.48	12.73	13.12	14.52	12.70	12.77	13.16	12.56	13.33	12.99	13.66	13.18	12.44	12.89	13.76	12.61	14.46
14	96.25	96.74	94.94	96.25	93.38	96.10	94.56	96.65	86.46	94.49	94.70	95.34	95.52	95.56	96.24	96.66	96.16	95.55
15	81.75	82.10	80.83	81.44	84.82	81.67	80.55	80.73	79.93	80.46	80.90	80.88	81.06	81.38	81.65	81.56	81.65	81.35
16	66.38	65.84	65.70	66.31	69.73	66.06	65.38	65.93	64.52	64.19	65.68	65.73	65.40	66.06	65.63	65.66	66.32	66.33
17	48.75	49.40	47.84	48.81	51.23	48.90	48.23	48.56	47.65	47.91	48.77	49.03	48.12	49.44	48.97	48.81	47.42	48.46
18	13.44	13.00	12.81	13.06	14.13	13.18	12.88	12.88	12.60	12.59	13.63	13.64	12.97	13.06	13.35	13.49	12.94	13.77
19	11.13	11.11	10.98	11.12	10.48	11.26	10.97	11.07	10.65	10.69	11.34	11.73	11.02	11.13	11.47	11.21	11.18	11.80
20	20.00	19.78	19.93	20.25	20.98	19.86	19.71	20.06	19.64	19.66	20.20	20.27	20.06	20.56	20.32	20.69	20.07	21.01
21	9.94	9.81	9.93	10.31	10.49	10.51	9.76	9.95	9.64	10.13	10.34	10.92	9.95	10.44	10.52	10.84	9.89	9.88
22	88.25	88.23	88.16	88.38	95.63	88.12	87.37	87.98	86.26	86.19	87.49	86.89	87.68	88.00	88.18	87.98	88.97	88.05
23	20.44	20.33	20.37	20.38	21.57	20.56	20.28	20.64	20.01	20.56	20.79	20.85	20.27	20.25	20.55	20.85	20.12	20.78
24	13.75	13.79	13.48	13.44	12.83	13.54	13.43	13.58	13.04	13.32	14.07	14.04	13.46	13.63	13.66	13.85	13.68	13.96
25	20.25	20.06	19.89	19.94	20.97	20.11	19.85	20.46	19.58	19.24	20.10	20.63	19.86	20.19	20.27	20.27	20.36	20.89
26	59.19	59.14	56.91	59.19	60.39	58.52	58.51	58.93	57.96	57.94	59.24	58.77	58.88	59.19	59.14	59.38	59.05	59.47
27	13.63	13.74	13.11	14.00	16.30	13.38	13.21	13.01	13.32	13.24	12.41	14.01	13.04	12.94	13.44	13.09	13.63	13.85
28	44.56	44.42	44.15	44.62	47.80	44.56	44.14	45.29	43.78	43.73	44.66	43.96	44.22	44.31	44.79	44.40	45.20	45.15
29	10.44	10.34	10.18	9.94	10.48	10.46	10.27	10.41	10.29	10.31	10.45	11.01	10.22	10.38	10.42	10.51	10.13	11.21
30	89.81	90.24	89.26	89.88	88.02	89.48	88.79	90.04	88.40	88.59	88.56	89.40	89.06	89.31	89.80	89.94	89.89	89.75

[a] Cochran rank sum procedure shows a significant difference at $P < 0.05$, and lab. × sample interaction calculation shows a significant difference at $P < 0.05$.

lumping all the variation into the repeatability estimate (s_r).

Each 1.0 mm pair has a complementary 0.5 mm pair, so it is possible to compare precision between methods for the 2 grinds (Table 5). There is noticeable improvement in precision for the combustion method when the sample is ground finer; this effect was expected.

Sample means for the 0.5 mm pairs are compared in Table 6. Differences in sample means, combustion−Kjeldahl, range from −0.12 to 0.63, with an average difference of 0.23% protein. Only pair 1, blood meal, shows a higher mean by the Kjeldahl method. The 2 methods produced results that were not significantly different according to paired *t*-tests (ref. 3, pp. 55–58) except for pair 5, meat and bone meal, and pair 10, feather meal.

Table 3. Performance of combustion analyzer method: determination of nitrogen in standards and in corn–soybean mixture ground to 0.5 mm (10 determination each in each laboratory)

	Nicotinic acid		Lysine·HCl		Corn–soybean mixture		
Lab.	% N, av.	SD	% N, av.	SD	% N, av.	SD	RSD, %
1	11.42	0.03	15.39	0.06	2.99	0.03	1.00
2	11.48	0.04	15.43	0.09	3.00	0.02	0.77
3	11.46	0.03	15.43	0.07	3.03	0.01	0.42
4	11.47	0.03	15.35	0.04	3.03	0.05	1.65
5	11.01	0.09	15.04	0.07	2.95	0.07	2.37
6	11.42	0.07	15.17	0.04	3.10	0.02	0.68
7	11.34	0.04	15.22	0.09	2.99	0.03	1.00
8	11.42	0.03	15.42	0.04	3.06	0.03	0.98
9	11.46	0.05	15.30	0.07	3.08	0.01	0.48
Theory	11.38		15.34				

The grand means of the 140 results by each method on the 0.5 mm samples were 47.41% protein by the Kjeldahl method and 47.65% protein by the combustion method. For the 1.0 mm samples, the grand means of the 56 results by each method were 31.50% protein by the Kjeldahl method and 31.82% protein by the combustion method.

Results on the standard materials (Table 7) show that recoveries were lower by the Kjeldahl method. Recoveries for 5 of the 9 laboratories were low on nicotinic acid and 2 had difficulty with lysine·HCl by the Kjeldahl method (data for laboratories 3 and 5 removed from statistical analysis). All results for the combustion method are satisfactory except perhaps the lysine value for laboratory 5.

Conclusions

The performance of the combustion method compares favorably with that for the mercury catalyst Kjeldahl method for determination of crude protein in feeds. Standard deviations by the combustion method covered the full range of possibilities compared to the Kjeldahl method; values were either equivalent, better than, or not as good as for the Kjeldahl method. For the 0.5 mm pairs, values ranged from 0.09 to 0.58% protein for the Kjeldahl method and from 0.14 to 0.33% protein for the combustion method, and (s_R) values ranged from 0.23 to 0.86% protein (Kjeldahl) and from 0.30 to 0.61% protein (combustion).

The recovery data on standards demonstrates the ease in

Table 4. Comparison of combustion and mercury catalyst Kjeldahl methods: analysis of pairs ground to 0.5 mm and to 1.0 mm

Sample pair	Mercury Kjeldahl method: Mean % protein X	Mercury Kjeldahl method: Mean % protein Y	s_r	s_L	s_R	Combustion method: Mean % protein X	Combustion method: Mean % protein Y	S_r	s_L	s_R
Samples ground to 0.5 mm										
7	11.16	10.30	0.11	0.13	0.21	11.31	10.54	0.14	0.35	0.52
9	13.65	13.15	0.13	0.24	0.36	13.76	13.27	0.15	0.25	0.39
4	15.69	15.38	0.09	0.19	0.28	16.05	15.59	0.17	0.26	0.41
8	20.40	20.08	0.21	0.07	0.23	20.58	20.35	0.16	0.25	0.39
2	46.70	44.53	0.26	0.29	0.49	46.80	44.59	0.30	0.29	0.51
5	51.68	48.30	0.52	0.06	0.52	52.11	48.93	0.28	0.15	0.35
6	65.78	58.70	0.58	0.29	0.71	65.98	59.15	0.19	0.16	0.30
3	84.64	81.22	0.46	0.51	0.86	85.02	81.67	0.23	0.40	0.61
10	89.31	81.20	0.17	0.48	0.69	89.79	81.34	0.31	0.26	0.47
1	88.33	88.01	0.42	0.48	0.80	88.21	87.93	0.33	0.31	0.55
						Pooled		0.28		0.52
						Pooled	RSD_r, %	0.59	RSD_R, %	1.10
Samples ground to 1.0 mm										
11	20.37	20.04	0.17	0.21	0.34	20.33	20.37	0.56	0.08	0.58
12	11.09	10.05	0.13	0.22	0.34	11.47	10.31	0.28	0.34	0.56
13	83.89	80.44	0.88	<0	0.79	84.26	80.89	0.86	<0	0.82
14	13.21	12.94	0.36	<0	0.35	13.52	13.44	0.41	0.38	0.68

[a] Data from laboratories 3 and 5 excluded.

Table 5. Comparison of combustion and Kjeldahl methods on 1.0 and 0.5 mm complementary samples

Approx. % protein	Material	Fineness, mm	Kjeldahl s_r	Kjeldahl s_L	Kjeldahl s_R	Combustion s_r	Combustion s_L	Combustion s_R
20	Corn–soybeans	1.0	0.17	0.21	0.34	0.56	0.08	0.58
		0.5	0.13	0.24	0.36	0.15	0.25	0.39
11	Swine diet	1.0	0.13	0.22	0.34	0.28	0.34	0.56
		0.5	0.11	0.13	0.21	0.14	0.35	0.52
84	Beef finish	1.0	0.88	<0	0.79	0.86	<0	0.82
		0.5	0.46	0.51	0.86	0.23	0.40	0.61
13	Swine finish	1.0	0.36	<0	0.35	0.41	0.38	0.68
		0.5	0.13	0.24	0.36	0.15	0.25	0.39

Table 6. Comparison of means by combustion and Kjeldahl methods on 0.5 mm samples

Sample pair	Sample No.	Mean % protein: Kjeldahl	Mean % protein: Combustion	Difference, Comb. − Kjel.
1	1	88.33	88.21	−0.12
	22	88.01	87.93	−0.08
2	3	46.70	46.80	0.10
	28	44.53	44.59	0.06
3	7	84.64	85.02	0.38
	12	81.22	81.67	0.45
4	8	15.69	16.05	0.36
	5	15.38	15.59	0.21
5	10	51.68	52.11	0.43**
	17	48.30	48.93	0.63**
6	16	65.78	65.98	0.20
	26	58.70	59.15	0.45
7	19	11.16	11.31	0.15
	29	10.30	10.54	0.24
8	23	20.40	20.58	0.18
	25	20.08	20.35	0.27
9	24	13.65	13.76	0.11
	18	13.15	13.27	0.12
10	30	89.31	89.79	0.48**
	15	81.20	81.34	0.14**

** Significant difference at $P = 0.05$ using paired t-test.

Table 7. Collaborative results for % crude protein[a] in standard materials

Lab.	Nicotinic acid: Kjeldahl	Nicotinic acid: Combustion	Lysine·HCl: Kjeldahl	Lysine·HCl: Combustion
1	71.63	70.38	96.25	96.74
2	71.21	71.21	94.94	96.25
3	63.58[b]	71.69	93.38[b]	96.10
4	68.29[b]	71.36	94.56	96.65
5	18.31[b]	70.42	86.46[b]	94.49
6	66.48[b]	71.64	94.70	95.34
7	55.71[b]	71.44	95.52	95.56
8	71.57	71.50	96.24	96.66
9	71.68	71.28	96.16	95.55
Av.	71.52	71.21	95.48	95.93
Theory	71.12		95.88	

[a] Crude protein = % N × 6.25.
[b] Excluded from average.

which the combustion method recovers nitrogen and emphasizes the difficulties of achieving full recovery with Kjeldahl methods. This has to be considered when comparing the 2 methods or making recommendations. The average difference between methods was 0.04% N.

The question of fineness of grind for samples for the com-

bustion method is best answered by performance criteria demonstrated in this study. The 0.5 mm sample grind met the performance guideline and was adequate for the combustion method as represented by the 2 LECO instruments. The traditional 1.0 mm grind did not perform as well. Sample size, which must be considered, ranged from 90 to 200 mg, with most weights in the 120–160 mg range. In the future, instruments that could accept a larger sample size should not be restricted to a sample fineness determined at a time when sample size was limited to the 100–200 mg range.

The generic presentation of the combustion method seems to have held up well in this study. The accuracy requirement was easily met by the different laboratories. Of the 2 laboratories which used the LECO CHN 600 instrument, results for one were dropped from the study, leaving only laboratory 6 using the CHN 600. The other laboratories (1, 2, 4, 7, 8, and 9) used the LECO FP-228.

Recommendation

It is recommended that the combustion method be adopted official first action with the following change in the performance guidelines on accuracy: The mean of the 10 determinations of nicotinic acid and the mean of the 10 determinations of lysine monohydrochloride must each be within ± 0.15 of the respective theory, with a standard deviation not to exceed 0.15. Standard tryptophan may be substituted for lysine monohydrochloride.

The combustion method should also include a statement on safety, such as, "Follow manufacturer's recommendations for safe operation of the instrument."

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R. D. Cathey, University of Missouri, Experiment Station Chemical Laboratories, Columbia, MO

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G. I. Wilkens, Agway, Inc., Technical Center, Ithaca, NY

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FOOD ADDITIVES

Determination of Organic Acids in the Presence of Metal Ion

MASAMICHI KOSEKI, IWAO TAKAHASHI, TETSUKO YASUNO, SHUZO OGINO, YUKO TSUDA, and MASAYOSHI KAZAMA
Tokyo Metropolitan Research Laboratory of Public Health, 24-1, Hyakunincho, 3-chome, Shinjuku-ku, Tokyo 169, Japan

The recovery of organic acids: citric, tartaric, fumaric, succinic, and malic, was examined by liquid chromatography in the presence of calcium, magnesium, or aluminum ions. Calcium and magnesium ions, i.e., ions of valence +2, did not interfere in determinations of the organic acids examined here. These organic acids could be determined without interference from aluminum ion by pH adjustment of the solutions to pH 2.

Liquid chromatography (LC) is generally used to determine organic acids in food additive preparations (1) and in foods (2). Enzymatic analysis can also be used (3). When citric acid was determined in the presence of aluminum ion, we found that the recovery of citric acid decreased for both LC and enzymatic analyses, but aluminum did not interfere when the pH of the sample solution was less than 3 (4). These results suggested that interaction between other organic acids and metal ions should occur.

We investigated the effect of calcium, magnesium, and aluminum ions on the determination of citric, tartaric, fumaric, succinic, and malic acids, and developed an LC method for determination of these organic acids in the presence of aluminum ion. The weight ratio of metal salts to organic acids in this experiment was set at 1:1 and 10:1, because the ratios of aluminum salts to organic acids in food additive preparations sold commercially correspond to the range between 1:1 and 7:1.

Experimental

Determination of Organic Acids

All organic acids were determined by both reverse-phase and ion exchange LC analysis using peak areas. Conditions for reverse-phase column: Nucleosil 7 C-18, 30 cm × 4 mm id; eluant, potassium dihydrogen phosphate water solution (2% w/v, pH 2.4); flow rate, 1.0 mL/min; volume of sample solution injected, 5 μL; detector, UV 210 nm (UVIDEC-100 V, Japan Spectroscopic, Ltd, Tokyo, Japan) (1). Conditions for ion exchange column: Shodex ionpack KC-8111, 30 cm × 8 mm id; eluant: 0.1% phosphoric acid solution; flow rate, 1.0 mL/min; volume of sample solution injected, 10 μL; detector, UV 210 nm.

Preparation of Organic Acid and Metal Ion Solutions

Control solutions were prepared by dissolving 50 mg citric acid monohydrate, tartaric acid, succinic acid, or malic acid, or 1 mg fumaric acid, respectively, in water. Sample solutions were prepared by adding 50 mg (1 mg fumaric acid) of $CaCl_2 \cdot 2H_2O$, $MgCl_2 \cdot 6H_2O$, or $K_2Al_2(SO_4)_4 \cdot 24H_2O$ to each control solution (low concentration) or 500 mg (10 mg fumaric acid) (high concentration). Finally, these solutions were diluted to 100 mL with water after adjustment from pH 2 to 8 with HCl or NaOH solutions. The molar ratios of metal ions to acids are shown in Table 1. The concentration of each organic acid in the solutions was determined by LC analysis. Recovery of organic acid was calculated by comparing the LC peak area of the acid in the sample solution with that of the control solution.

Results

Effect of Calcium and Magnesium Ions

Recoveries of citric, tartaric, fumaric, succinic, or malic acids in the pH range between 2 and 8 were 100% when the concentration of calcium or magnesium ions was either high or low. Therefore, the presence of calcium or magnesium ions does not interfere.

Effect of Aluminum Ions

The low concentration of aluminum ion had no effect on the determination of citric acid. When the ratio of aluminum ion to citric acid was 10:1, no interference was observed if the pH was less than 3 (Figure 1A).

Low concentrations of aluminum ion did not interfere in the determination of tartaric acid in all pH ranges studied. At a ratio of 10:1, the interference of aluminum ion was severe except at pH 2 (Figure 1B).

The effect of aluminum ion on the determination of fumaric acid was very slight. Even at 10:1, it had minimal effect at all pH ranges studied (Figure 1C).

The recovery of succinic acid was 100% at pH ranges less than 4 for both low and high concentrations of aluminum ion (Figure 1D).

The recovery of malic acid was 100% except for slight interference at pH 4 and 5 for low concentrations of aluminum ion. For high concentrations of aluminum ion, recovery decreased rapidly at the pH ranges higher than 4 but recovery was 100% at pH 2 (Figure 1E).

Figure 2 shows the LC chromatograms of citric acid in the presence or absence of aluminum ion. Peak broadening or skewing was not observed. Peak shapes of other organic acids were not different from those of citric acid.

Experiments with Ion Exchange LC

The recovery of organic acids in the presence of aluminum ion was not improved by ion exchange LC (Figure 1), although peak shapes from the ion exchange chromatogram were not affected.

Discussion

The recovery of citric acid by LC and enzymatic analysis decreased in the presence of food additive preparations. The

Table 1. Molar ratios[a] of metal ions to acids in test solutions

Organic acid	Calcium ion	Magnesium ion	Aluminum ion
Citric	1.43	1.03	0.44
Tartaric	1.02	0.74	0.32
Fumaric	0.79	0.57	0.24
Succinic	0.80	0.58	0.25
Malic	0.91	0.66	0.28

[a] Experiments at 10:1 metal ion and acid ratios were also done.

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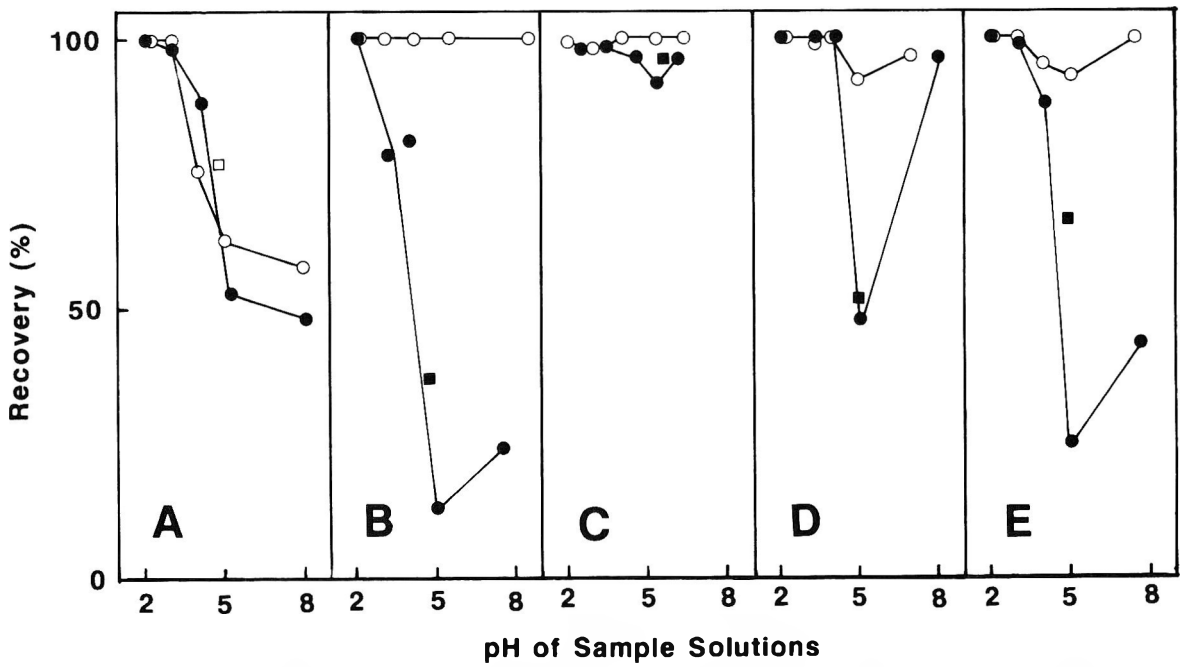

Figure 1. Recovery of organic acids in presence of aluminum ion at pH range between 2 and 8. For reverse-phase LC, ratio of $K_2Al_2(SO_4)_4 \cdot 24H_2O$ to acid was 1:1 (○) and 10:1 (●). For ion exchange LC, ratio of $K_2Al_2(SO_4)_4 \cdot 24H_2O$ to acid was 1:1 (□) and 10:1 (■). A, Citric acid; B, tartaric acid; C, fumaric acid; D, succinic acid; E, malic acid.

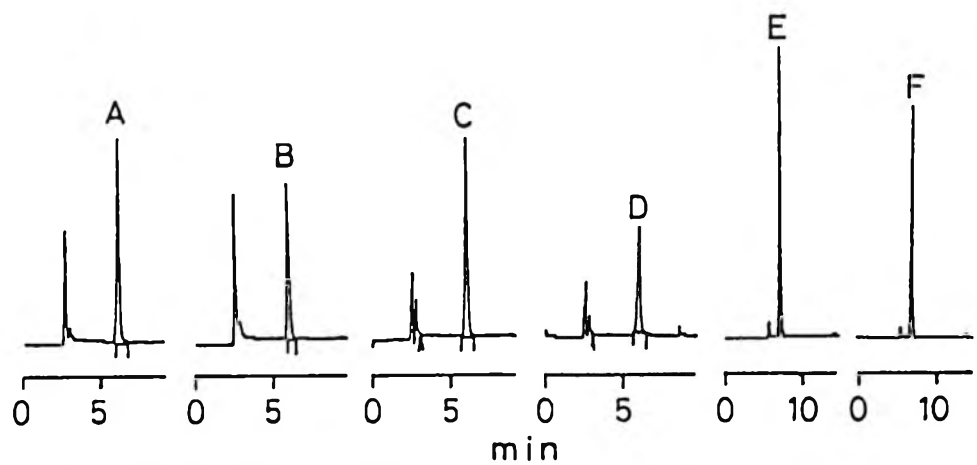

Figure 2. Chromatograms of A, C, reverse-phase LC of citric acid without aluminum ion; B, ratio of $K_2Al_2(SO_4)_4 \cdot 24H_2O$ to citric acid 1:1; D, ratio 10:1; E, chromatograms of ion exchange LC of citric acid without aluminum ion; F, ratio of $K_2Al_2(SO_4)_4 \cdot 24H_2O$ to citric acid 1:1.

result of orthogonal L_{27} design, which is a statistical technique to determine the significant factor among many factors affecting the sample (5), showed that the recovery of citric acid had been affected by aluminum ion in sample solutions above pH 3 (4). Therefore, the determination of other organic acids was examined in the presence of metal ion. Calcium and magnesium ions did not influence recovery. Aluminum ion suppressed recovery of organic acids, but recovery returned to 100% when pH of the sample solutions was adjusted.

To test whether the occurrence of aluminum/acids complexes might reduce the recovery of organic acids by reverse-phase LC in higher pH ranges, organic acids were determined by ion exchange LC in the presence of aluminum ion. However, the recovery of organic acids was not improved, probably because the bonding between aluminum ion and acids was greater than that between aluminum ion and ion exchange resin. The LC retention times of aluminum/acid complexes should be different from those of free acids and should be much longer; the sample peak shapes were the same as the control, and no other peaks were found.

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MEATS AND MEAT PRODUCTS

Homogeneity of Meats Prepared for Analysis with a Commercial Food Processor: Collaborative Study

ROBERTA M. BEEBE, ERIC LAY, and SYLVAN EISENBERG[1]
Anresco, Inc., 1370 Van Dyke Ave, San Francisco, CA 94124

Collaborators: F. Braggs; P. Chang; D. Egelhofer; D. Howard; M. A. Jacobs; E. Lay; W. G. Miller; R. Sellards; A. Tandoc; N. V. Yong; R. H. Albert (*Statistical Consultant*)

The food processor was evaluated as an alternative to the food chopper and bowl cutter for preparing meat samples for analysis in AOAC procedure 24.001. Samples of 6 meat types—cooked sausage, pork sausage, canned ham, hamburger, water-added ham, and smoked ham—were distributed to 9 laboratories for preparation using a food processor. The resulting 54 samples were sent to a USDA-accredited laboratory for analysis in triplicate for moisture, protein, and fat. Standard deviations and their 95% confidence intervals calculated for the analytical results were compared with USDA Performance Standards. With few exceptions, the upper limits were lower than the Performance Standards and for the exceptions, the intervals included the Performance Standards. By these criteria, the food processor is as effective in preparing homogeneous samples as the preparation procedures used to set the Performance Standards. Collaborators found the processor faster to use and easier to clean than the food chopper. Use of the food processor has received interim approval as an alternative to the food chopper or bowl cutter in AOAC procedure 24.001 for preparing meat samples for analysis.

Preparation of meat samples for analysis both by AOAC procedure **24.001** (1) and by USDA procedure 1.002B (2) requires passing the sample 3 times through a food chopper with plate openings $\leq 1/8$ in. The AOAC procedure further requires "mixing thoroughly after each grinding," whereas the USDA procedure requires "careful mixing with a spatula between each chopping." The USDA procedure further specifies that "all sample chopping is to be performed using clean and dry equipment which is at room-temperature." It adds, "High fat meat samples, i.e., bacon, pork, sausage, must be nearly frozen before chopping." The new AOAC bowl cutter procedure **24.001(a)** (3) requires that all cutter parts be chilled before preparation of each sample. The background study for use of the bowl cutter (4) specified that all frozen samples and any cutter parts that may contact them were tempered in a −1.5 to −2.0°C cold room overnight.

In the present study of the food processor, we tried to minimize use of subjective terms such as "mixing thoroughly," and to avoid requirements that are difficult to implement in practice, such as precooling equipment and samples to below 0°C. The food processor differs from either the food chopper or bowl cutter in principle; it is a batch operation. The sample is confined to the mixing chamber with its cover and is exposed only to the cutting knives. These are easy to clean and dry between samples. In our experience the food processor not only chops and blends hard materials such as pepperoni to make a fine homogeneous granular mix, but also emulsifies phases that have separated as a result of storage or thawing. As used here, the word homogeneous requires definition. A bulk sample from which subsamples are to be taken at random for analysis is homogeneous if such subsamples are so alike analytically that they differ from each other only within the USDA Performance Standards (5). This requirement becomes increasingly demanding as the subsample size is decreased.

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The recommendation has received interim approval of the General Referee, the Committee on Foods I, and the Chairman of the Official Methods Board. The procedure will be submitted for adoption at the 103rd AOAC Annual International Meeting, September 25–28, 1989, at St. Louis, MO. Association actions will be published in "Changes in Official Methods of Analysis" (1990) *J. Assoc. Off. Anal. Chem.* **73**, January/February issue.
[1] Associate Referee.

Collaborative Study

Preparation of Samples

Twenty pounds each of cooked sausage (hot dogs), pork sausage (links), hamburger, canned ham, water-added ham, and smoked ham were obtained from a local wholesaler. Each was divided as randomly as possible into 2 lb units and transferred to nine 1 gal. zip-lock freezer bags. The sausages were transferred as units. Other meats were cut into slices about $1/2$–$3/4$ in. thick that were then cut into 1 to 2 in. lengths before packaging, one unit in the first bag, then a second in the second bag, and so on until each of 9 bags was filled. Contents were so arranged that bags could be flattened before freezing. The frozen samples, one of each variety, were packed in insulated containers (No. 355, Polyfoam Packers Corp., 2320 S Foster Ave, Wheeling, IL 60090). An extra bag of cooked sausage was included for pretrial use by each collaborator. An extra bag of hamburger was included in the set for laboratory 7, this to be prepared for analysis using the food chopper. Six refrigerant packs (No. 420, Polyfoam U-Tek) were put in each container, 3 on bottom and 3 on top. A rubber straight-edged household spatula was included.

This method of randomizing bulk samples of intrinsically nonhomogeneous materials differs from that used by Pettinati et al. (4) who prepared samples by first course-grinding them through a food chopper with a $1/2$ in. plate. Weber and Edelson (6) produced uniform nonhomogeneous samples of butter by distributing pats of 6 different butters in different jars in sequence much as we distributed pieces into bags.

The containers were sent to each of the collaborators either by local messenger service, by bus, or by an air delivery service as required so that samples were delivered frozen.

Instructions to Collaborators

Participants were instructed to prepare the samples according to the procedure and to repack the processed samples in properly labeled zip-lock bags, flatten the bags, freeze the samples, and then transfer the processed, frozen samples to the original shipping containers with the original, refrozen refrigerant packs. The containers were then to be shipped to laboratory 5 or to us for transshipment to laboratory 5 where all analytical work was to be done. Laboratory 5 received all processed samples frozen, and retained them frozen until they were thawed prior to subsampling for analysis. Samples processed at laboratory 5 were not frozen and thawed prior to subsampling. This distinction is important because it exposed a problem in thawing frozen samples before subsampling.

Table 1. Proximate analysis (% fat, moisture, and protein) of cooked sausage, pork sausage, and hamburger prepared for subsampling in food processor

	Cooked sausage			Pork sausage			Hamburger		
Lab.	Fat	Moisture	Protein	Fat	Moisture	Protein	Fat	Moisture	Protein
1	27.4	52.7	12.8	28.8	55.0	12.9	15.7	65.4	18.2
	27.7	52.8	13.3	28.8	55.1	13.2	15.4	65.6	18.4
	27.6	52.7	13.1	29.3	54.9	13.5	16.0	65.7	18.2
2	27.5	53.0	13.0	29.6	53.6	12.9	16.0	63.9	18.6
	27.5	53.3	13.0	29.5	54.5	13.0	17.2	63.7	18.3
	27.5	52.9	13.0	29.8	55.1	13.7	17.7	64.8	17.7
3	26.9	53.4	13.3	29.0	54.9	13.4	16.5	64.6	18.2
	26.9	53.6	13.1	29.4	55.2	13.2	16.6	64.7	18.0
	26.7	53.6	13.2	28.8	55.2	13.4	16.5	64.6	17.9
4	26.4	54.6	12.7	29.2	55.1	13.2	16.6	66.1	18.2
	26.8	54.7	12.6	29.5	54.7	13.2	16.3	66.4	18.3
	26.9	54.3	13.1	29.4	55.0	13.5	15.3	65.2	18.2
5	27.4	52.8	13.3	30.1	53.9	12.8	16.5	65.5	18.2
	27.4	52.8	13.1	30.7	54.4	12.8	15.7	65.4	18.5
	27.7	52.9	12.9	30.8	53.8	13.0	15.6	65.9	18.5
6	27.7	53.0	13.0	29.8	54.5	13.1	16.7	65.3	17.6
	27.1	52.9	12.9	29.8	54.4	13.4	16.2	65.1	18.1
	27.2	52.7	13.0	29.5	54.4	13.3	15.5	64.8	18.5
7	27.1	53.3	13.1	29.5	54.7	13.1	15.1	65.8	18.2
	27.4	53.2	13.0	29.0	55.0	13.3	16.0	65.5	18.1
	27.1	52.9	13.1	29.0	54.9	13.1	14.7	65.5	18.3
8	27.3	53.2	13.1	29.8	54.4	13.6	14.9	66.3	18.6
	27.4	53.2	13.1	29.0	54.4	13.5	14.8	66.5	18.5
	27.3	53.5	13.2	28.7	54.6	13.1	14.3	66.5	18.5
9	27.3	53.3	12.9	29.7	54.3	13.6	15.7	65.4	18.1
	27.3	53.1	13.3	29.5	53.8	13.5	16.1	65.4	17.9
	27.5	53.1	13.1	28.9	54.3	13.4	15.9	65.3	18.0

Table 2. Proximate analysis (% fat, moisture, and protein) of canned ham, water-added ham, and smoked ham prepared for subsampling in food processor

	Canned ham			Water-added ham			Smoked ham		
Lab.	Fat	Moisture	Protein	Fat	Moisture	Protein	Fat	Moisture	Protein
1	1.5	74.6	18.5	3.5	74.4	17.7	7.5	67.5	16.7
	1.5	74.1	18.4	3.5	74.4	18.1	7.4	67.3	17.2
	1.5	74.6	18.5	3.7	74.5	17.4	7.0	67.5	16.9
2	1.8	74.7	18.4	3.0	75.9	16.8	7.0	68.2	17.1
	1.8	74.4	18.5	2.8	75.8	17.3	7.0	68.4	17.2
	1.7	74.6	18.4	2.7	75.5	17.1	6.6	68.3	16.8
3	1.8	74.5	18.5	2.8	74.8	18.2	8.0	66.8	17.4
	1.7	74.7	18.7	2.8	74.6	18.5	7.7	66.7	17.8
	1.7	74.4	18.8	2.8	74.5	18.5	7.7	66.8	17.4
4	1.7	74.8	18.4	3.2	76.2	17.4	8.1	67.2	16.7
	1.6	74.6	18.5	3.2	75.7	17.5	8.1	67.2	16.5
	1.6	74.6	18.4	3.1	75.2	16.8	8.3	67.3	16.5
5	1.9	74.2	18.5	3.0	75.3	17.4	7.0	67.8	17.3
	2.0	74.2	18.8	3.2	75.2	17.7	7.3	68.0	16.8
	1.9	74.3	18.5	3.1	75.6	17.4	7.1	68.0	16.9
6	1.4	74.7	18.5	2.8	75.8	17.6	8.9	66.8	16.3
	1.4	74.8	18.6	2.7	75.6	16.9	8.6	66.9	16.2
	1.4	74.8	18.4	2.7	75.5	17.4	8.6	67.0	16.3
7	2.1	74.3	17.9	2.5	75.7	17.5	7.6	67.2	16.5
	2.1	74.4	18.0	2.9	75.3	17.7	7.5	67.3	16.7
	1.8	74.5	17.7	2.9	75.6	17.6	8.0	67.3	16.9
8	1.5	75.1	18.1	2.7	75.4	17.2	8.3	67.0	16.9
	1.5	74.7	18.2	3.0	75.4	17.2	8.2	67.0	17.0
	1.8	74.8	18.6	3.2	75.5	17.1	8.3	67.1	16.8
9	2.0	75.1	17.1	3.4	75.1	16.7	7.9	67.3	16.6
	2.0	75.0	17.9	3.2	75.1	16.8	7.7	67.4	17.0
	2.0	74.9	17.7	3.1	75.1	16.1	7.6	67.2	17.0

Table 3. Variance for each set of triplicate analyses in Table 1

	Cooked sausage			Pork sausage			Hamburger		
Lab.	Fat	Moisture	Protein	Fat	Moisture	Protein	Fat	Moisture	Protein
1	0.0233	0.0033	0.0633	0.0833	0.0100	0.0900	0.0900	0.0233	0.0133
2	0.0000	0.0433	0.0000	0.0233	0.5700	0.1900	0.7633	0.3433	0.2100
3	0.0133	0.0133	0.0100	0.0933	0.0300	0.0133	0.0033	0.0033	0.0233
4	0.0700	0.0433	0.0700	0.0233	0.0433	0.0300	0.4633	0.3900	0.0033
5	0.0300	0.0033	0.0400	0.1433	0.1033	0.0133	0.2433	0.0700	0.0300
6	0.1033	0.0233	0.0033	0.0300	0.0033	0.0233	0.3633	0.0633	0.2033
7	0.0300	0.0433	0.0033	0.0833	0.0233	0.0133	0.4433	0.0300	0.0100
8	0.0033	0.0300	0.0033	0.3233	0.0133	0.0700	0.1033	0.0133	0.0033
9	0.0133	0.0133	0.0400	0.1733	0.0833	0.0100	0.0400	0.0033	0.0100
Pooled									
SD^2	0.0318	0.0240	0.0259	0.1087	0.0978	0.0503	0.2792	0.1044	0.0563
SD	0.178	0.155	0.161	0.330	0.313	0.244	0.528	0.323	0.237

Table 4. Variance for each set of triplicate analyses in Table 2

	Canned ham			Water-added ham			Smoked ham		
Lab.	Fat	Moisture	Protein	Fat	Moisture	Protein	Fat	Moisture	Protein
1	0.0000	0.0833	0.0033	0.0133	0.0033	0.0233	0.0700	0.0133	0.0633
2	0.0033	0.0233	0.0033	0.0233	0.0433	0.0633	0.0533	0.0100	0.0433
3	0.0033	0.0233	0.0233	0.0000	0.0233	0.0300	0.0300	0.0033	0.0533
4	0.0033	0.0133	0.0033	0.0033	0.2500	0.1433	0.0133	0.0033	0.0133
5	0.0033	0.0033	0.0300	0.0100	0.0433	0.0300	0.0233	0.0133	0.0700
6	0.0000	0.0033	0.0100	0.0033	0.0233	0.1300	0.0300	0.0100	0.0033
7	0.0300	0.0100	0.0233	0.0533	0.0433	0.0100	0.0700	0.0033	0.0400
8	0.0300	0.0433	0.0700	0.0633	0.0033	0.0033	0.0033	0.0033	0.0100
9	0.0000	0.0100	0.1733	0.0233	0.0000	0.1433	0.0233	0.0100	0.0533
Pooled									
SD^2	0.0081	0.0237	0.0378	0.0215	0.0481	0.0752	0.0352	0.0078	0.0389
SD	0.090	0.154	0.194	0.147	0.219	0.274	0.188	0.088	0.197

Table 5. 95% Confidence limits (18 degrees of freedom) for pooled standard deviations

Meat type	Fat	Moisture	Protein
Cooked sausage	0.135–0.263	0.117–0.229	0.122–0.238
Pork sausage	0.250–0.488	0.237–0.463[a]	0.169–0.332
Hamburger	0.399–0.781	0.244–0.478	0.179–0.351
Canned ham	0.068–0.133	0.116–0.228	0.147–0.287
Water-added ham	0.111–0.218	0.166–0.324[b]	0.207–0.406
Smoked ham	0.142–0.278	0.067–0.130	0.149–0.292
USDA Performance Standards:			
Repeatability	<0.63	<0.46	<0.24
Reproducibility	<0.66	<0.65	<0.32

[a] With Lab. 2 outlier omitted (16 df): 0.147–0.300.
[b] With Lab. 4 outlier omitted (16 df): 0.112–0.230.

Subsampling for Analysis

Thawed samples were flattened, then subsamples were taken geometrically rather than at random by removing them along a diagonal, one at the corner, one midway to center, and one at the center of bag. Each subsample was analyzed for fat, moisture, and protein by AOAC methods **24.005(a), 24.003(a),** and **24.027,** respectively (1).

Preliminary evaluation of the data for laboratories 1–4 of the 9 participating laboratories as well as the subsampling procedure itself disclosed a serious problem. Phase separation occurred when the samples were thawed, and the phases could not be homogenized simply by kneading the 2 lb samples in their respective bags. Normally, phase separation is not a problem because samples are not usually frozen before subsampling for analysis. But if samples are frozen, phase separation must be addressed.

The subsampling protocol was changed so that thawed samples were returned to the food processor, and the phases were recombined by processing for 30 s. Samples were then returned to their respective bags, were flattened, and subsamples were removed as before. Analyses for laboratories 1–4 were repeated.

Statistical Treatment

One USDA-accredited laboratory was selected to do all the analytical work in order to avoid interlaboratory systematic errors. Homogeneity of processed samples was estimated by 3 parameters, repeatability of triplicate fat, moisture, and protein determinations. Fluctuations in composition reflect both the random discontinuities in composition and the random within-laboratory errors. Estimates are therefore maximum errors, testing more severely than necessary.

Results and Discussion

Samples Thawed and Then Reprocessed

Samples processed at laboratory 5 were not refrozen after processing; those samples were not reprocessed for subsampling. Processed samples sent to laboratory 5 for analysis by the other collaborators were thawed and then reprocessed 30 s before subsampling. Results for all laboratories are reported in Tables 1 and 2. Corresponding variances for each set of triplicates are reported in Tables 3 and 4 as are pooled

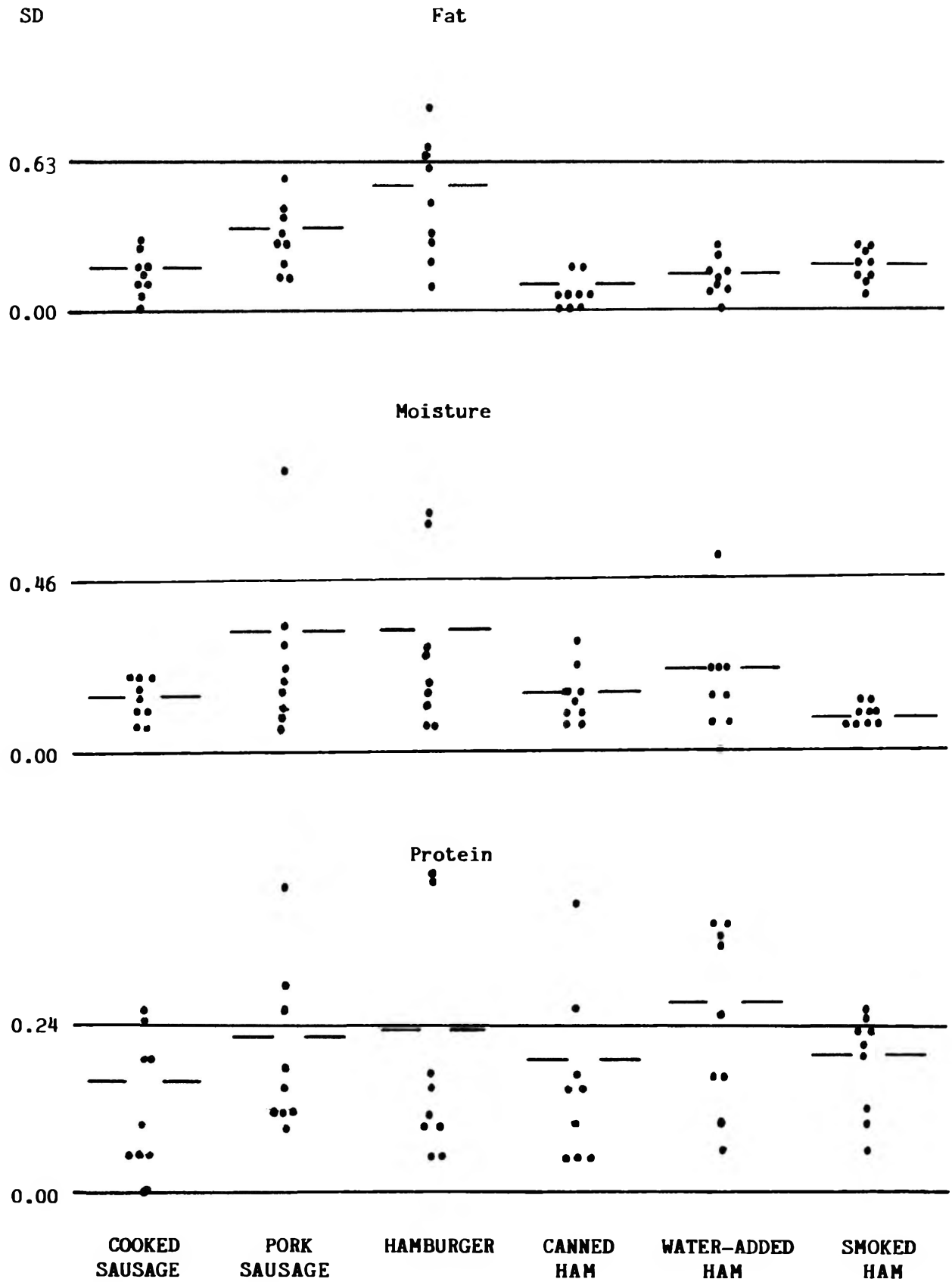

Figure 1. Scattering of standard deviations for triplicate analyses of 6 meat types processed by 9 laboratories. Upper solid lines for fat, moisture, and protein represent the USDA Repeatability Performance Standards. Broken lines represent pooled standard deviations (reproducibility among laboratories).

standard deviations and variances. Figure 1 illustrates scattering of the individual standard deviations. Table 5 reports the 95% confidence limits for these standard deviations for analysis of the 6 meat types for fat, moisture, and protein. These limits were calculated as suggested by Wernimont (ref. 7, p. 166). The factors for the lower and upper limits for 18 degrees of freedom are, respectively, 0.76 and 1.48. We note that the limits found either include or are lower than the

Table 6. Proximate analysis (% fat, moisture, and protein) of cooked sausage, pork sausage, and hamburger: terminal 30 s processing after thawing omitted

	Cooked sausage			Pork sausage			Hamburger		
Lab.	Fat	Moisture	Protein	Fat	Moisture	Protein	Fat	Moisture	Protein
1	27.6	52.9	12.8	29.5	55.0	13.6	15.6	65.7	17.6
	27.8	53.7	12.9	29.1	54.6	13.5	16.4	65.1	17.9
	27.7	53.3	12.7	29.0	54.9	13.4	15.2	65.1	18.2
2	—[a]	52.8	12.5	—	54.5	12.9	—	65.2	18.1
	—	53.7	12.5	—	54.6	13.0	—	64.7	18.4
	—	54.1	12.6	—	54.6	13.0	—	66.8	17.6
3	26.3	53.8	12.8	28.9	55.7	13.0	15.0	64.9	17.4
	26.2	54.3	12.7	28.7	54.7	12.7	14.4	65.0	16.6
	26.8	53.3	12.7	29.9	55.0	13.0	16.7	64.3	17.8
4	27.8	53.7	12.6	29.6	54.5	13.1	14.8	65.5	17.6
	26.0	55.1	12.5	29.5	54.5	13.0	15.2	67.3	17.7
	26.3	55.2	12.4	29.2	54.7	13.0	15.6	64.0	16.9

[a] Procedure changed to include 30 s reprocessing before these fat analyses were performed.

Table 7. Proximate analysis (% fat, moisture, and protein) of canned ham, water-added ham, smoked ham: terminal 30 s processing after thawing omitted

	Canned ham			Water-added ham			Smoked ham		
Lab.	Fat	Moisture	Protein	Fat	Moisture	Protein	Fat	Moisture	Protein
1	1.6	74.4	17.9	3.3	75.8	17.1	7.5	68.6	16.0
	1.7	75.1	18.0	3.3	75.6	17.4	7.3	68.0	16.7
	1.7	74.8	18.1	3.6	74.9	17.0	7.6	67.5	17.2
2	—[a]	74.8	18.0	—	75.8	16.5	—	67.9	15.8
	—	74.8	17.9	—	77.0	16.2	—	68.1	17.2
	—	74.4	18.3	—	75.3	16.8	—	68.0	17.0
3	1.7	74.7	18.8	2.8	76.0	17.3	8.0	67.0	16.0
	1.8	74.5	16.5	2.9	76.9	18.2	8.4	66.6	17.8
	1.9	74.5	18.6	3.2	75.2	18.3	9.5	67.1	18.2
4	1.5	74.9	18.3	3.0	75.3	17.4	7.0	67.8	17.3
	1.7	74.6	18.6	3.2	75.2	17.7	7.3	68.0	16.8
	1.6	74.5	18.8	3.1	75.6	17.4	7.1	68.0	16.9

[a] See Table 6.

respective USDA Performance Standards. The food processor produced samples either more homogeneous or as homogeneous as those produced by the method on which the Performance Standards are based.

Evaluation would be even more favorable if 2 outliers among the 162 triplicate analyses were excluded. Moisture values in pork sausage processed by laboratory 2 and in water-added ham processed by laboratory 4 are outliers as judged by testing homogeneity of variation from ranges (ref. 7, p. 155).

Samples Kneaded in Bags After Thawing

Table 6 and 7 report proximate analyses in triplicate for samples processed by laboratories 1–4. Data in Tables 6 and 7 are values for samples kneaded in their bags after thawing at the laboratory doing the analyses. Those workers had trouble reincorporating fluid that had separated on thawing. Corresponding variances and pooled variances for those data are shown in Tables 8 and 9. When the protocol was changed to reprocess thawed samples for 30 s, analyses showed marked improvement in homogeneity of all meat types other than pork sausage, as judged by the variance ratios that are also shown in Tables 8 and 9. Pork sausage analyses were satisfactory by either treatment. When ratios of the variances in Tables 8 and 9 to those in Tables 3 and 4 (the F-statistic) were compared with tabulated values of $F_{0.95}$ (ref. 7, p. 150), 10 of the 18 F-values greatly exceeded the tabular values. One may conclude with confidence that reprocessing thawed samples for a short time is much more effective than kneading.

Hamburger Prepared for Analysis by Two Methods

Table 10 reports proximate analyses in triplicate for 2 bulk samples of hamburger, one prepared for analysis with the food processor and the other with the food chopper. Samples were prepared by a laboratory experienced with the food chopper so that we could expect the prepared sample to be "practically" homogeneous. These data serve no statistical function in this study, but are included to provide comparative information concerning the 2 pieces of equipment.

General comments about the food processor follow. However, one comment about the food chopper and the hamburger is particularly important and not often appreciated: "After each of the three passages, fat had to be removed from the barrel immediately behind the plate with a rubber spatula and mixed into the sample by hand. This fat removal required partial disassembly." The corresponding comment about the food processor was that "The ground beef sample put through the food processor deposited large amounts of fat on the bowl wall and the blades. The scraping off of the fat with a rubber spatula and working it back into the meat by hand was judged more laborious than with the chopper. Removing the cover was the only disassembly required."

We note that the instructions for processing samples do not include working the fat back into the meat by hand. Rather, the scrapings are transferred to the body of the meat and processing is continued for 30 s.

Table 8. Variances for each set of triplicate analyses in Table 6 (no reprocessing after thawing) compared to those of Table 3 (30 s reprocessing after thawing)

	Cooked sausage			Pork sausage			Hamburger		
Lab.	Fat	Moisture	Protein	Fat	Moisture	Protein	Fat	Moisture	Protein
1	0.0100	0.1600	0.0100	0.0700	0.0433	0.0100	0.3733	0.1200	0.0900
2	—	0.4433	0.0033	—	0.0033	0.0033	—	1.2033	0.1633
3	0.1033	0.2500	0.0033	0.4133	0.2633	0.0300	1.4233	0.1433	0.3733
4	0.9300	0.7033	0.0100	0.0433	0.0133	0.0033	0.1600	2.7300	0.1900
Pooled									
SD^2	0.3478	0.3892	0.0067	0.1755	0.0808	0.0117	0.6533	1.0492	0.2042
df	6	8	8	6	8	8	6	8	8
F^a	10.8	16.2	<1	1.6	<1	<1	2.33	10.0	3.63
$F_{0.95}{}^b$	2.7	2.5	2.5	2.7	2.5	2.5	2.7	2.5	2.5

[a] Ratio of corresponding variances, no reprocessing (above) to 30 s reprocessing (Table 3; df = 18).

[b] From ref. 7 (p. 150, Table A-5), upper limits for variance ratios reached by chance 1 in 20 times if both variances are really equal.

Table 9. Variances for each set of triplicate analyses in Table 7 (no reprocessing after thawing) compared to those of Table 4 (30 s reprocessing after thawing)

	Canned ham			Water-added ham			Smoked ham		
Lab.	Fat	Moisture	Protein	Fat	Moisture	Protein	Fat	Moisture	Protein
1	0.0033	0.1233	0.0100	0.0300	0.2233	0.0433	0.0233	0.3033	0.3633
2	—	0.0533	0.0433	—	0.7633	0.0900	—	0.0100	0.5733
2	0.0100	0.0133	1.6233	0.0433	0.7233	0.3033	0.6033	0.0700	1.3733
3	0.0100	0.0433	0.0633	0.0100	0.0433	0.0300	0.0233	0.0133	0.0700
Pooled									
SD^2	0.0078	0.0583	0.4349	0.0278	0.4383	0.2775	0.2166	0.0992	0.5948
df	6	8	8	6	8	8	6	8	8
F^a	<1	2.46	11.5	1.29	9.11	3.69	6.15	12.7	15.3
$F_{0.95}{}^b$	2.7	2.5	2.5	2.7	2.5	2.5	2.7	2.5	2.5

[a] Ratio of corresponding variances, no reprocessing (above) to 30 s reprocessing (Table 4; df = 18).

[b] From ref 7 (p. 150, Table A-5), upper limits for variance ratios reached by chance 1 in 20 times if both variances are really equal.

Table 10. Proximate analysis (% fat, moisture, and protein) of hamburger prepared for analysis by food processor and by food chopper by laboratory 7, samples of like origin but not necessarily alike analytically

	Food processor			Food chopper		
Statistic	Fat	Moisture	Protein	Fat	Moisture	Protein
	15.1	65.8	18.2	14.0	66.4	18.1
	16.0	65.5	18.1	14.8	66.4	17.8
	14.7	65.5	18.3	13.8	67.1	18.1
Av.	15.27	65.60	18.20	14.20	66.63	18.00
SD	0.67	0.17	0.10	0.53	0.40	0.17
CV, %	4.4	0.03	0.55	3.7	0.61	0.96
Sum[a]		99.17			98.83	

[a] Sum of fat, moisture, and protein.

Comments of Participating Laboratories

Eight of the 9 participants returned general comments. All agreed that the food processor was much easier to clean than the food chopper. Seven participants considered it easier to use; 4 said it did a better job of homogenizing; 3 considered it faster; and one commented that, compared to the food chopper, there was essentially no loss of sample.

There were also a number of specific comments: For pork sausage, 4 reported that a small amount of product accumulated around the motor shaft. For hamburger, 5 reported problems with fat accumulated on side walls of the processor and the need to reincorporate the material. Another participant with considerable experience commented that hamburger was always a problem and that the food processor was the best equipment he and his 5 co-workers had used.

Conclusion

The food processor homogenizes various meat types at least as well as or better than required to meet USDA Performance Standards. Particular care must be taken with certain meat types, particularly ground beef, to assure uniform distribution of fat. The food processor is easier to clean and is generally faster than the food chopper. It is less expensive than either the bowl cutter or the food chopper specified by Pettinati et al. (4).

Recommendation

We recommend that the food processor be accepted as an alternative to the food chopper specified in **24.001** for preparation of meat and meat products for analysis.

Revise **24.001(a)** to add an alternative to the food chopper and bowl cutter, as follows:

Alternatively, use food processor (benchtop model, 110/120 V, 60 Hz, 1 hp, 7.5 A, 1725 rpm, fan-cooled motor, 4 qt bowl, Model R4Y, Robot Coupe, U.S.A., Inc., Jackson, MS, or equivalent). *Caution*: Do not remove cutter bowl lid or cutter bowl from base until motor has come to full stop. Do not put hand, finger, or any object into bowl while motor is running. Unplug appliance before servicing or cleaning.

Precut up to 2 lb sample to maximum dimension ≤ 2 in., and transfer to bowl for processing. Include any separated liquid. Process 30 s, then wipe down inner side wall and

bottom of bowl with spatula (use household plastic or rubber spatula with ca 2 in. by 4 in. straight-edge blade) and transfer gathered material to body of sample. Continue processing another 30 s and wipe down as before. Repeat sequence to give total of 2 min processing and 3 wipe downs.

Take particular care with certain meat types such as ground beef to assure uniform distribution of fat and connective tissue. At each wipe-down interval, reincorporate these into sample by using spatula to remove fat from inside surfaces of bowl and connective tissue from around blades. If sample consolidates as ball above blades, interrupt processing and press sample to bottom of bowl with spatula before continuing.

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METHOD PERFORMANCE

Precision Parameters of Standard Methods of Analysis for Dairy Products

JAMES T. PEELER
Food and Drug Administration, 1090 Tusculum Ave, Cincinnati, OH 45226
WILLIAM HORWITZ and RICHARD ALBERT
Food and Drug Administration, Center for Food Safety and Applied Nutrition, Washington, DC 20204

The available collaborative studies for standard methods of analysis for various constituents of milk and milk products were examined in an attempt to assign specific repeatability and reproducibility precision parameters to these methods. The different collaborative assays for the primary constituents (moisture/solids, fat, protein), the nutritionally important elements (calcium, sodium, potassium, phosphorus), and miscellaneous analytes/physical constants (ash, lactose, salt, freezing point) produced different estimates of the precision parameters for the same method. A suitable summary of the precision estimates from collaborative studies is given by the reproducibility relative standard deviation, RSD_R, which is relatively constant within a product and permits comparisons across products. An estimate of the variation of RSD_R for an analyte from a number of collaborative studies is presented in terms of the median and 90% interval (the range of the centermost 90% of values). These estimates are only informative when a substantial number of independent studies are available for pooling the independent estimates to form a distribution of RSD_R values. The RSD_R for the determination of the primary constituents of milk and milk products is characterized by a median RSD_R of 1% and a 90% interval of 0.3–3%, with RSD_R estimates occasionally occurring below 0.3% and above 4%. These overall estimates appear to be independent of analyte, matrix, and method and apply to concentrations of primary constituents that range from about 2 to 80%. The repeatability relative standard deviation, RSD_r, is unstable, although it tends to converge to about 0.5–0.7 × RSD_R. Too few collaborative assays are available to characterize RSD_R for the determination of certain other constituents (acidity, ash, lactose, salt, and the nutritionally important elements) unless RSD_R values for different analytes, methods, and matrixes are pooled on the basis of similar analyte concentrations. When pooled, the RSD_R values are generally better than predicted from the Horwitz equation, RSD_R (%) = $2 \exp(1 - 0.5 \log_{10} C)$, where C is the concentration expressed as a decimal fraction; all but one of 661 RSD_R values are within the upper empirical limit of twice this curve.

The American Public Health Association (APHA) publishes a number of methods books including *Standard Methods for the Examination of Dairy Products* (SMEDP) (1). Ginn et al. (2) have noted that all new methods considered for acceptance by the APHA Committee for future editions of SMEDP must undergo and successfully pass the AOAC collaborative study procedure. This requirement does not imply that all new methods must be adopted by AOAC. However, it does mean that the collaborative study protocol must be followed, including the presentation of performance parameters (3). Such adherence to protocol is a necessary but not sufficient requirement for method approval.

New methods included in SMEDP will include precision estimates. Many of the older studies of methods, including methods already adopted by AOAC, do not have precision estimates, or if such estimates were made, the size of the study may not meet current requirements. In some cases only averages, results from an analysis of variance, or summary data were published. The chemical methods for dairy products constitute more than half of the quantitative procedures in SMEDP. The objective of the present study was to calculate the precision parameters on a consistent, acceptable statistical basis for those chemical methods for dairy products (2) for which data were available.

The following performance parameters are recommended (3) for incorporation into *Official Methods of Analysis* (4): "percent recovery; reproducibility coefficient of variation (CV_x) at the appropriate concentration level; minimum detectable level for the analyte (method sensitivity); applicable concentration range; repeatability (within laboratory) standard deviation (S_o); among laboratory standard deviation (S_l); reproducibility (including repeatability) standard deviation (S_x)." The symbols and terminology were modified slightly in subsequent actions by AOAC, i.e., by the use of relative standard deviation (RSD) for coefficient of variation (CV) and the use of r and R as subscripts to indicate the corresponding repeatability and reproducibility statistics. The purpose for the inclusion of S_l (now S_L) is not clear since it serves only as an intermediate quantity for the estimation of other parameters that have more practical significance.

All of the currently recommended parameters are characteristic of descriptive statistics intended to summarize data—in this case, to summarize the results of collaborative studies. However, what is really desired is the application of the results of collaborative studies to the quality control of similar individual analyses conducted in the future. The application of quality control-type statistics to future individual analyses requires a probability statement with respect to the distribution of future values, e.g., 95% of the time a future value would be expected to lie between specific limits as a function of the mean and standard deviation (or its related measures such as RSD). Recognition of this difference resulted in the formation of a new working group of the International Union of Pure and Applied Chemistry (IUPAC) to determine the best way to report the results of collaborative studies. The repeatability (r) and reproducibility (R) values of the International Organization for Standardization (ISO) are the only parameters based on this concept in practical analytical work. Repeatability and reproducibility values indicate how close individual results should be to each other when produced by the same analyst within a given laboratory (r) and by single analysts in different laboratories (R), respectively (5). Statistically, these parameters are known as maximum tolerable differences, defined typically for the 95% probability (P) level as $2 \cdot 2^{1/2} \cdot S_{(r \text{ or } R)}$. For quality control work it is the *distribution* of the standard deviation, S, and of r, R, and RSD_R values that is of critical interest, not just the long-term averages of these values. Furthermore, the existence of a hierarchy of precision estimates should be noted: the precision estimate of a method of analysis as applied by a given analyst in a study (r-terms); the precision estimate of the method across laboratories in a given study (R-terms); and the precision estimate across all studies involving the method. Practically no attention has been given to the last and broadest precision estimate in the hierarchy.

In this examination we have abandoned the conventional first approximation that a given method under fixed conditions has a unique, characteristic precision. We concede that

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the underlying precision does change with circumstances, but we have attempted here to present an empirical distribution of RSD_R. Such a description inevitably confounds the variability of the underlying precision with the variability due to sampling of the performance of laboratories (actually analysts). Previously this variability had been ascribed entirely and arbitrarily to method performance rather than to the premise that a substantial but unknown proportion is due to laboratory (or analyst) performance.

These statistical considerations are greatly simplified in an examination of the analytical data from dairy products. The composition of these products has been regulated for almost a century. Consequently, the concentration of each analyte for each product is more or less fixed.

Procedure

The chemical methods in SMEDP (1), the corresponding methods in *Official Methods of Analysis* (4), and the supporting references, primarily from the latter, are listed in Table 1. To save space, references throughout are given a nonredundant format of "(year of publication), first page of the article in 4 digits for computer sorting, and a final set of 3 letters for computer retrieval of the raw data and identification information," e.g., (1988)0893PBJ. Similarly, AOAC method numbers in the text are given as "section number and [last 2 digits of year of publication]," e.g., 2.055[84]. References that did not contain collaborative data are suffixed as "NoD"; if fewer than 5 laboratories supplied data, the actual number of laboratories (L) is indicated. Data from such limited studies were not ordinarily incorporated into our compilation unless they were part of a large study or were about the only data available. Many of the references contained only results of intralaboratory work, method descriptions or revisions, or recommendations. In addition, data from some collaborative studies published in the AOAC *Journal* but not referenced in *Official Methods of Analysis* and data from recently published collaborative studies conducted by the International Dairy Federation (IDF) (6) were incorporated into the database.

The original raw data from references containing collabo-

Table 1. Chemical methods listed in *Standard Methods for the Examination of Dairy Products* (SMEDP) (1), *Official Methods of Analysis* (OMA) (4), and references in AOAC *Journal* (JAOAC) unless otherwise indicated

Item no.	Analyte/method/matrix	SMEDP No. (class)[a]	OMA sec.[b]	JAOAC ref.[c]
1	Acidity, titratable/ titrimetric/milk	18.3.A(0)	16.023	(1947)0130NoD (1951)0239PAA
2	Ash/gravimetric/	18.4.A(0)	16.035	(1940)0453PAG
	milk, evaporated		16.190	(1945)0211NoD
3	Chloride (salt)/Mohr/butter	18.5.A(0)	16.235	(1951)0239PAA (1966)0518PAW
4	Chloride (salt)/Volhard/	18.5.B(A1)		(1941)0100,0631PAB
	cheese		16.272	(1982)1350PBB (1935)0401NoD (1937)0339PAX
	Chloride (salt)/potentiometric/ cheese	18.5.C(B)	16.268	(1982)1350PBB
5	Extraneous matter/ microscopic/cheese	18.7(0)	44.022	(1967)0501 L = 2
6	Fat/Babcock/milk	18.8.A(0)	16.065	(1924)0004NoD
	milk, homogenized			(1967)0537PAN
	milk, whole raw			(1972)0488PAM
	milk, raw unpreserved			(1973)0140PAL
	milk, raw unpreserved			(1975)0949PAD
7	Fat/Roese-Gottlieb method (ether extraction)/various	18.8.E(0)	16.064	(1951)0236NoD (1969)0235NoD
	milk, homogenized			(1967)0537PAN
	milk, chocolate			(1967)0537PAN
	milk, dried		16.219	IDF Bul. 235(1988)0068 (1927)0308PBL (1932)0524PAZ (1969)0240NoD
	milk, evaporated		16.192	(1945)0207 L = 2 (1969)0239NoD
	milk, sweetened condensed		16.205	(1945)0207 L = 2 (1969)0239NoD
	(Schmid-Bondzynski)/cheese		16.284	(1933)0584PBC (1948)0300PBV (1949)0303PBV
	whey cheese		16.285	(1974)0264NoD
	ice cream		16.316	(1952)0212NoD
7A	Fat/rapid detergent/		16.067*	(1967)0537PAN
	milk, homogenized		16.055(11th)	
	milk, chocolate		(surplus)	
8	Fat/automated turbidity Milko-testerFOSS)/	18.8.G(A1)	16.068	(1969)0131 L = 2 (1975)0572NoOD
	milk			(1980)0238NoD (1980)0713 L = 1 (1980)0716NoD

Table 1. Continued

Item no.	Analyte/method/matrix	SMEDP No. (class)[a]	OMA sec.[b]	JAOAC ref.[c]
9B	Fat, moisture, and salt/ Kohman/butter	18.9.C(0)	—	(1969)0235,0394NoD
	Fat/indirect/butter	—	16.232	(1938)0361NoD (1952)0194PBD
9C	Lactose/polarimetric/milk	—	16.055	(1942)0603NoD
	enzymatic	—	16.059	(1944)0232NoD (1984)0637PBG
9	Multicomponent/infrared/milk	18.9.A(A1)	16.083	(1972)0488PAM
	fat		16.086	(1978)1015PAC
	protein		16.088	(1979)1202 L = 1
	lactose		16.090	(1979)1211NoD
	solids		16.092	(1980)0973 L = 1
9A	AutoAnalyzer(Technicon)/ milk	18.9.B(A2)	16.074	(1973)1401PAL
10	Moisture/solids/vacuum oven/ cheese	18.10.A(0)	16.259	(1926)0044NoD (1935)0057NoD
	Solids/vacuum oven/evaporated milk	—	16.189	(1940)0453PAG (1945)0211NoD
	Solids/vacuum oven/dried milk	—	16.212	(1927)0308PBL (1928)0289 L = 2
	Solids/gravimetric/ice cream	18.10.A(0)	16.313	(1941)0575NoD
11	Moisture/air-oven/ cheese	—	16.260	(1948)0300PAV (1949)0303PAV
	/distillation/cheese	—	16.261	(1969)0117PBE
12	Moisture/microwave oven/ cheese	18.10.D (A1, A2, B)	16.265	(1977)1392PAE JFP[d](1984)0272
—	Acidity and ash/milk			(1951)0239PAA
—	Preparation of sample	—	16.187	(1945)0207NoD (1969)0239NoD
13	Protein/Kjeldahl/ milk	18.11.A (A1)	16.036	(1955)0056NoD (1969)0126PAP (1988)0893PBJ
14	Protein/dye-binding, Amido black/milk	18.11.B (A2)	16.042	(1974)1338NoD (1975)0770PAI
15	Protein/dye-binding, Acid orange-12/milk	18.11.C (A2)	16.037	(1967)0542NoD (1967)0557 L = 1 (1968)0811PAF (1969)0138PAK (1975)0773PAJ
16	Added water/cryoscope (thermistor)/milk	18.12.A (A1)	16.096	(1961)0438 L = 1 (1968)0816PAH (1970)0539NoD (1972)0504NoD
17	Added water/vapor pressure osmometer/milk	18.12.B (A2)	16.101	(1978)1038NoD

[a] Key to SMEDP classification:
0 = Widely used, but no collaborative study (Grandfathered Standard Methods.)
A1 = Extensively used and collaboratively studied.
A2 = Collaboratively studied.
B = Used in research, not collaboratively studied.
C = Proposed.
D = Previously approved, but superseded.
0, A1, A2 are "Standard Methods."

[b] * = AOAC surplus method.

[c] References are given in a nonredundant form of (year) first page in 4 digit format. Letter suffixes refer to string labels in $WAH/PEELER (beginning P) or in $WAH/BERT for cross-referencing to database. L = number of laboratories. NoD = No collaborative data; NoOD = No original data.

[d] *Journal of Food Protection.*

rative studies were analyzed by an updated version of the program FDACHEMIST (7), and the statistical parameters of laboratory mean ($\bar{x}$) and relative standard deviations within-laboratories (RSD_r) and between-laboratories (including within-) (RSD_R) were calculated assay-by-assay ("sample"-by-"sample"). The updated version of the program permits the flagging of all outliers ($P = 0.01$) and stragglers ($P = 0.05$), but removes outliers only by the IUPAC-1987 protocol (Cochran, Grubbs, and paired-Grubbs outlier tests, applied in that order, at a probability of 0.01) (8). However, these signals were occasionally arbitrarily overridden for the present compilation when removal of the outlier failed to change the RSD_R estimate by a meaningful amount, or when the RSD_R was already consistent with the other values in the same study. Similarly, an occasional obvious "chemical" outlier was removed, although it was flagged only as a straggler. As pointed out by Taylor (9), the purpose of outlier removal is not to improve the statistics, but to discover potential

problems with the methods. Consequently, few outliers were removed from the reported data, and the outlier history, although retrievable through rerunning the program, was not entered into the database. Only the number of laboratories removed (usually 0) and the total number of laboratories reporting valid data were tabulated.

This recalculation from the raw data with a single, accepted statistical procedure resulted in a comparable set of estimates of statistical parameters for further examination. Since the precision parameters for IDF studies (6) had already been calculated in the same way as calculated by FDACHEMIST, with and without outliers removed, the reported values could be used directly.

A few papers contained precision parameters from an analysis of variance (ANOVA) but did not contain the raw data. Since the statistical parameters calculated from the ANOVA treatment were comparable to those obtained by recalculation with raw data presented in other studies of the same or similar analyte-matrix-method combinations, these ANOVA-calculated parameters were included in the database. When only a few assays (e.g., 4) were available, as in the case of acidity, the precision parameters were included in the database, although the number of RSD_R values available for interpretation were not considered sufficient to provide a reasonable estimate of the dispersion of the precision parameters.

A few studies reported only the average for a number of replicates. The correction factor to a single value basis is obtained by dividing by a factor which is a function of the number of replicates (k) that were averaged, assuming $S_r = 0.5\ S_R$, which is consistent with our experience. This correction factor is $[(3/4) + (1/4k)]^{1/2}$, so that the RSD_R value calculated from the average is divided by 0.935 or $(7/8)^{1/2}$ for duplicates, 0.913 or $(5/6)^{1/2}$ for triplicates, and 0.901 or $(13/16)^{1/2}$ for quadruplicates, to obtain the corrected RSD_R value. Since this factor at worst is about 10% and the studies requiring correction are so few, the program was not revised to handle this situation.

Results

Presentation.—The identifying and characterizing parameters for each collaborative assay (record) were automatically entered into a database of 12 fields similar to that published for drug substances (10). Since a standard treatment for outlier identification and removal is now available and since outlier removal was not important in this data set, a number of the fields defined in the original database were not used here.

The database of 661 accepted collaborative assays of materials (Database 1) was prepared, arranged alphabetically by analyte, by matrix within analyte, by method within matrix, and in increasing order of analyte concentration. A material is the homogeneous commodity (matrix) subdivided into identical test samples and distributed to the collaborating laboratories. The calculated results of the collaborative assays are given by average concentration "CONCN," usually in g/100 g ("PCT."), and by the precision parameters RSD_r ("RSD0" was substituted in the output because of printer limitations) and RSD_R ("RSDR"). The "DROPPED/# LABS" column shows the number of outliers removed by the IUPAC-1987 criterion (often 0)/original number of laboratories reporting valid data. The "CODE" column provides the key to retrieval of the raw data from the computer. The bibliographic section under the head "METHOD NO." gives the section number and the year of publication for AOAC methods. The section number in early

Database 1. Database of accepted assays for standard methods for examination of dairy products

OBS	ANALYTE	MATRIX	METHOD	CONCN	UNITS	RSD0	RSDR	DROPPED/ # LABS	CODE	METHOD NO.	REFERENCE	HORRAT
1	ACIDITY	MILK	VOLU	0.315		0.87	0.87	0/ 5	AA1A	<16.023 84 >	.FRARY.34,.239.(1951).	.
2	ACIDITY	MILK	VOLU	0.283		0.00	1.44	1/ 5	AA1AB	<16.023 84 >	.FRARY.34,.239.(1951).	.
3	ACIDITY	MILK	VOLU	0.316		1.16	1.35	0/ 5	AA2A	<16.023 84 >	.FRARY.34,.239.(1951).	.
4	ACIDITY	MILK	VOLU	0.280		1.29	1.52	0/ 5	AA2AB	<16.023 84 >	.FRARY.34,.239.(1951).	.
5	ASH	CHEESE	GRAV	3.415	PCT.	0.49	0.95	0/11	AX01A	<16.267 84 >	.STONE.20,.339.(1937).	0.28573
6	ASH	MILK	GRAV	1.458	PCT.	0.82	3.80	0/ 5	AA2BB	<16.035 84 >	.FRARY.34,.239.(1951).	1.00549
7	ASH	MILK	GRAV	1.460	PCT.	0.62	2.97	0/ 5	AA2B	<16.035 84 >	.FRARY.34,.239.(1951).	0.78603
8	ASH	MILK	GRAV	1.530	PCT.	0.47	1.57	0/ 5	AA1B	<16.035 84 >	.FRARY.34,.239.(1951).	0.41847
9	ASH	MILK	GRAV	1.531	PCT.	0.45	1.78	0/ 5	AA1BB	<16.035 84 >	.FRARY.34,.239.(1951).	0.47449
10	ASH	MILK-EVA	GRAV	1.691	PCT.	1.71	3.50	0/ 8	AG1E	<XXII49 35 >	.FRARY.23,.453.(1940).	0.94696
11	ASH	MILK-EVA	GRAV	1.702	PCT.	0.72	2.19	0/ 7	AG2D	<XXII9 35 >.	.FRARY.23,.453.(1940).	0.59313
12	ASH	MILK-EVA	GRAV	1.704	PCT.	0.54	2.03	0/ 7	AG1D	<XXII9 35 >.	.FRARY.23,.453.(1940).	0.54987
13	ASH	MILK-EVA	GRAV	1.706	PCT.	0.61	1.35	1/ 8	AG2E	<XXII49 35 >	.FRARY.23,.453.(1940).	0.36577
14	CALCIUM	MILK-DRI	ATAB	0.921	PCT.	2.02	2.54	1/12	AY03C1	<IDF36:1966R	.LEENHER.IDFBUL235,0034(1988)	0.62715
15	CALCIUM	MILK-DRI	ATAB	0.938	PCT.	2.51	4.09	0/12	AY03C2	<IDF36:1966R	.LEENHER.IDFBUL235,0034(1988)	1.01265
16	CALCIUM	MILK-DRI	ATAB	1.130	PCT.	1.30	2.54	1/12	AY02B2	<IDF36(1966R	.LEENHER.IDFBUL235,0034(1988)	0.64678
17	CALCIUM	MILK-DRI	ATAB	1.158	PCT.	0.55	2.05	2/12	AY02B1	<IDF36:1966R	.LEENHER.IDFBUL235,0034(1988)	0.52394
18	CALCIUM	MILK-DRI	ATAB	1.257	PCT.	1.50	3.06	1/12	AY01A2	<IDF36:19766	.LEENHER.IDFBUL235,0034(1988)	0.79178
19	CALCIUM	MILK-DRI	ATAB	1.262	PCT.	2.00	2.98	1/12	AY01A1	<IDF36(1966R	.LEENHER.IDFBUL235,0034(1988)	0.77157
20	CHLORIDE	CHEESE	VOLU	0.990	PCT.	0.51	1.20	0/ 8	BB01B	<16.272 84 >	.POORTVLIET.65,.1350.(1982).	0.29955
21	CHLORIDE	CHEESE	VOLU	1.002	PCT.	1.16	2.02	1/ 8	BB01A	<16.272 84 >	.POORTVLIET.65,.1350.(1982).	0.50516
22	CHLORIDE	CHEESE	VOLU	1.013	PCT.	0.43	1.69	0/ 8	BB01C	<16.268 84 >	.POORTVLIET.65,.1350.(1982).	0.42333
23	CHLORIDE	CHEESE	VOLU	1.404	PCT.	1.46	1.99	0/11	AX01B	<16.272 84 >	.STONE.20,.339.(1937).	0.52359
24	CHLORIDE	CHEESE	VOLU	1.475	PCT.	0.31	1.95	1/ 8	BB02B	<16.272 84 >	.POORTVLIET.65,.1350.(1982).	0.51687
25	CHLORIDE	CHEESE	VOLU	1.479	PCT.	0.72	1.85	0/ 8	BB02A	<16.272 84 >	.POORTVLIET.65,.1350.(1982).	0.49055
26	CHLORIDE	CHEESE	VOLU	1.504	PCT.	0.47	1.60	0/ 8	BB02C	<16.268 84 >	.POORTVLIET.65,.1350.(1982).	0.42533
27	CHLORIDE	CHEESE	VOLU	1.806	PCT.	0.48	1.47	0/ 8	BB03B	<16.272 84 >	.POORTVLIET.65,.1350.(1982).	0.40170
28	CHLORIDE	CHEESE	VOLU	1.809	PCT.	0.50	2.54	0/ 8	BB03A	<16.272 84 >	.POORTVLIET.65,.1350.(1982).	0.69428
29	CHLORIDE	CHEESE	VOLU	1.855	PCT.	0.33	0.97	0/ 8	BB03C	<16.268 84 >	.POORTVLIET.65,.1350.(1982).	0.26613
30	CHLORIDE	STZN	VOLU	0.100		0.07	0.13	0/ 6	AB1A	<MOHR>.....	.DEAL.24,.631.(1941).	.
31	CHLORIDE	STZN	VOLU	0.100		0.11	0.17	0/ 6	AB1B	<50.028 84 >	.DEAL.24,.631.(1941).	.
32	CHLORIDE	STZN	VOLU	0.100		0.07	0.10	0/ 6	AB1C	<50.030 84 >	.DEAL.24,.631.(1941).	.
33	CHLORIDE	STZN	VOLU	0.102		0.00	2.60	1/ 6	AB1D	<50.030 84 >	.DEAL.24,.631.(1941).	.
34	FAT	BUTTER	GRAV	81.171	PCT.	0.06	0.56	0/ 6	BD1C	<16.232 84 >	.WEBER.35,.194.(1952).	0.27134
35	FAT	BUTTER	GRAV	81.205	PCT.	0.05	0.31	0/ 6	BD2C	<16.232 84 >	.WEBER.35,.194.(1952).	0.15022
36	FAT	BUTTER	GRAV	81.252	PCT.	0.05	0.23	0/ 6	BD5C	<16.232 84 >	.WEBER.35,.194.(1952).	0.11146
37	FAT	BUTTER	GRAV	81.278	PCT.	0.07	0.25	0/ 6	BD4C	<16.232 84 >	.WEBER.35,.194.(1952).	0.12116
38	FAT	BUTTER	GRAV	81.291	PCT.	0.09	0.25	1/ 6	BD3C	<16.232 84 >	.WEBER.35,.194.(1952).	0.12116
39	FAT	BUTTER	GRAV	81.466	PCT.	0.06	0.48	0/ 6	BD6C	<16.232 84 >	.WEBER.35,.194.(1952).	0.23271
40	FAT	CHEESE-C	GRAV	30.855	PCT.	0.33	1.64	0/ 5	AV01D	<16.284 84 >	.HORWITZ.31,.300.(1948).	0.68699
41	FAT	CHEESE-C	GRAV	30.889	PCT.	0.76	1.42	0/ 5	AV01C	<16.284 84 >	.HORWITZ.31,.300.(1948).	0.59493
42	FAT	CHEESE-C	GRAV	31.918	PCT.	0.51	1.54	0/ 5	AV03D	<16.284 84 >	.HORWITZ.31,.300.(1948).	0.64840
43	FAT	CHEESE-C	GRAV	32.070	PCT.	0.54	1.21	0/ 5	AV03C	<16.284 84 >	.HORWITZ.31,.300.(1948).	0.50982
44	FAT	CHEESE-C	GRAV	34.923	PCT.	0.22	1.47	0/ 5	AV02D	<16.284 84 >	.HORWITZ.31,.300.(1948).	0.62736
45	FAT	CHEESE-C	GRAV	35.007	PCT.	0.44	1.53	0/ 5	AV02C	<16.284 84 >	.HORWITZ.31,.300.(1948).	0.65321
46	FAT	CHEESE-S	GRAV	30.259	PCT.	0.42	1.38	0/11	BC01B	<16.284 84 >	.LEPPER.16,.584.(1933).	0.57638
47	FAT	CHEESE-S	GRAV	30.400	PCT.	.	1.10	0/10	BC01A	<16.284 84 >	.LEPPER.16,.584.(1933).	0.45976
48	FAT	MILK	AUTO	3.027	PCT.	.	0.71	0/ 6	AL02B	<16.074 84 >	.KING.56,.1401(1973).	0.20970
49	FAT	MILK	AUTO	3.049	PCT.	.	1.82	0/ 5	AL02C	<16.074 84 >	.KING.56,.1401(1973).	0.53813
50	FAT	MILK	AUTO	3.147	PCT.	.	0.61	0/ 6	AL01B	<16.074 84 >	.KING.56,.1401(1973).	0.18122
51	FAT	MILK	AUTO	3.166	PCT.	.	1.86	0/ 5	AL01C	<16.074 84 >	.KING.56,.1401(1973).	0.55307
52	FAT	MILK	AUTO	3.333	PCT.	.	0.60	0/ 6	AL03B	<16.074 84 >	.KING.56,.1401(1973).	0.17980
53	FAT	MILK	AUTO	3.348	PCT.	.	0.77	0/ 6	AL05B	<16.074 84 >	.KING.56,.1401(1973).	0.23090

OBS	ANALYTE	MATRIX	METHOD	CONCN	UNITS	RSDO	RSDR	DROPPED/ # LABS	CODE	METHOD NO.	REFERENCE	HORRAT
54	FAT	MILK	AUTO	3.365	PCT.	.	1.99	0/ 5	AL03C	<16.074 84 >	.KING.56,.1401(1973).	0.597195
55	FAT	MILK	AUTO	3.367	PCT.	.	2.52	0/ 5	AL05C	<16.074 84 >	.KING.56,.1401(1973).	0.756321
56	FAT	MILK	AUTO	3.428	PCT.	.	0.83	0/ 6	AL06B	<16.074 84 >	.KING.56,.1401(1973).	0.249779
57	FAT	MILK	AUTO	3.447	PCT.	.	1.84	0/ 5	AL06C	<16.074 84 >	.KING.56,.1401(1973).	0.554185
58	FAT	MILK	AUTO	3.723	PCT.	.	0.59	0/ 6	AL04B	<16.074 84 >	.KING.56,.1401(1973).	0.179772
59	FAT	MILK	AUTO	3.734	PCT.	.	1.61	0/ 5	AL04C	<16.074 84 >	.KING.56,.1401(1973).	0.490772
60	FAT	MILK	AUTO	3.785	PCT.	.	0.76	0/ 6	AL07B	<16.074 84 >	.KING.56,.1401(1973).	0.232151
61	FAT	MILK	AUTO	3.806	PCT.	.	1.33	0/ 5	AL07C	<16.074 84 >	.KING.56,.1401(1973).	0.406600
62	FAT	MILK	AUTO	3.819	PCT.	.	1.15	0/ 6	AL09B	<16.074 84 >	.KING.56,.1401(1973).	0.351747
63	FAT	MILK	AUTO	3.828	PCT.	.	0.89	0/ 6	AL12B	<16.074 84 >	.KING.56,.1401(1973).	0.272314
64	FAT	MILK	AUTO	3.843	PCT.	.	1.98	0/ 5	AL09C	<16.074 84 >	.KING.56,.1401(1973).	0.606197
65	FAT	MILK	AUTO	3.855	PCT.	.	0.78	0/ 5	AL12C	<16.074 84 >	.KING.56,.1401(1973).	0.238917
66	FAT	MILK	AUTO	4.000	PCT.	.	1.20	0/ 6	AL10B	<16.074 84 >	.KING.56,.1401(1973).	0.369614
67	FAT	MILK	AUTO	4.019	PCT.	.	1.18	0/ 5	AL10C	<16.074 84 >	.KING.56,.1401(1973).	0.363701
68	FAT	MILK	AUTO	4.214	PCT.	.	0.59	0/ 6	AL08B	<16.074 84 >	.KING.56,.1401(1973).	0.183158
69	FAT	MILK	AUTO	4.219	PCT.	.	2.09	0/ 5	AL08C	<16.074 84 >	.KING.56,.1401(1973).	0.648909
70	FAT	MILK	AUTO	4.578	PCT.	.	1.30	0/ 5	AL11C	<16.074 84 >	.KING.56,.1401(1973).	0.408628
71	FAT	MILK	AUTO	4.581	PCT.	.	1.12	0/ 6	AL11B	<16.074 84 >	.KING.56,.1401(1973).	0.352077
72	FAT	MILK	AUTO	4.630	PCT.	.	0.63	0/ 6	AL13B	<16.074 84 >	.KING.56,.1401(1973).	0.198365
73	FAT	MILK	AUTO	4.656	PCT.	.	0.76	0/ 5	AL13C	<16.074 84 >	.KING.56,.1401(1973).	0.239495
74	FAT	MILK	AUTO	4.660	PCT.	.	0.62	0/ 6	AL15B	<16.074 84 >	.KING.56,.1401(1973).	0.195404
75	FAT	MILK	AUTO	4.682	PCT.	.	0.43	0/ 5	AL15C	<16.074 84 >	.KING.56,.1401(1973).	0.135618
76	FAT	MILK	AUTO	5.385	PCT.	.	0.82	0/ 6	AL14B	<16.074 84 >	.KING.56,.1401(1973).	0.264125
77	FAT	MILK	AUTO	5.447	PCT.	.	0.70	0/ 5	AL14C	<16.074 84 >	.KING.56,.1401(1973).	0.225858
78	FAT	MILK	GRAV	1.994	PCT.	1.92	1.92	0/ 6	AN06A	<16.064 84 >	.MITCHELL.50,.537.(1967).	0.532549
79	FAT	MILK	GRAV	1.995	PCT.	0.74	1.48	0/ 6	AN08A	<16.064 84 >	.MITCHELL.50,.537.(1967).	0.410533
80	FAT	MILK	GRAV	3.284	PCT.	0.54	0.92	0/ 6	AN04A	<16.064 84 >	.MITCHELL.50,.537.(1967).	0.275079
81	FAT	MILK	GRAV	3.294	PCT.	0.63	1.41	0/ 6	AN01A	<16.064 84 >	.MITCHELL.50,.537.(1967).	0.421782
82	FAT	MILK	GRAV	3.579	PCT.	0.20	0.98	0/ 6	AN05A	<16.064 84 >	.MITCHELL.50,.537.(1967).	0.296837
83	FAT	MILK	GRAV	3.769	PCT.	0.55	1.08	0/ 6	AN07A	<16.064 84 >	.MITCHELL.50,.537.(1967).	0.329684
84	FAT	MILK	GRAV	3.775	PCT.	0.17	0.72	0/ 6	AN03A	<16.064 84 >	.MITCHELL.50,.537.(1967).	0.219840
85	FAT	MILK	GRAV	3.898	PCT.	0.32	0.96	0/ 6	AN02A	<16.064 84 >	.MITCHELL.50,.537.(1967).	0.294542
86	FAT	MILK	INFR	2.672	PCT.	.	0.97	0/ 5	AM04B	<16.083 84 >	.BIGGS.55,.488.(1972).	0.281162
87	FAT	MILK	INFR	2.685	PCT.	1.06	2.38	0/ 6	AC01FI	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.690366
88	FAT	MILK	INFR	2.712	PCT.	0.37	2.74	0/ 6	AC01FC	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.795974
89	FAT	MILK	INFR	2.712	PCT.	0.93	2.39	1/ 6	AC01FH	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.694312
90	FAT	MILK	INFR	2.725	PCT.	0.70	1.53	0/ 6	AC01FD	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.444796
91	FAT	MILK	INFR	2.730	PCT.	0.75	2.05	0/ 6	AC01FB	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.596133
92	FAT	MILK	INFR	2.736	PCT.	0.16	1.49	1/ 6	AC01FE	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.433430
93	FAT	MILK	INFR	2.757	PCT.	0.39	2.29	0/ 6	AC04FC	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.666899
94	FAT	MILK	INFR	2.759	PCT.	0.46	2.68	0/ 6	AC04FI	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.780582
95	FAT	MILK	INFR	2.761	PCT.	0.67	1.06	0/ 6	AC04FH	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.308766
96	FAT	MILK	INFR	2.762	PCT.	0.40	2.20	0/ 6	AC04FE	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.640893
97	FAT	MILK	INFR	2.795	PCT.	0.69	2.67	1/ 6	AC04FD	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.779181
98	FAT	MILK	INFR	2.797	PCT.	0.53	1.58	0/ 6	AC04FB	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.461130
99	FAT	MILK	INFR	2.843	PCT.	1.04	1.83	0/ 6	AC11FF	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.535425
100	FAT	MILK	INFR	2.855	PCT.	0.76	1.75	1/ 6	AC11FJ	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.512335
101	FAT	MILK	INFR	2.886	PCT.	1.03	1.45	0/ 6	AC14FJ	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.425193
102	FAT	MILK	INFR	2.891	PCT.	0.19	2.54	1/ 6	AC14FF	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.745021
103	FAT	MILK	INFR	2.944	PCT.	0.51	1.28	0/ 6	AC09FJ	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.376475
104	FAT	MILK	INFR	2.950	PCT.	0.78	2.39	0/ 6	AC09FF	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.703158
105	FAT	MILK	INFR	2.967	PCT.	0.87	1.90	0/ 6	AC10FF	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.559470
106	FAT	MILK	INFR	2.985	PCT.	0.58	1.11	0/ 6	AC10FJ	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.327152
107	FAT	MILK	INFR	3.090	PCT.	0.61	2.10	0/ 6	AC02FI	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.622165
108	FAT	MILK	INFR	3.092	PCT.	0.48	1.81	0/ 6	AC02FC	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.536290
109	FAT	MILK	INFR	3.106	PCT.	0.45	2.36	0/ 6	AC26FK	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.699733
110	FAT	MILK	INFR	3.123	PCT.	0.98	1.55	0/ 6	AC02FH	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.459959
111	FAT	MILK	INFR	3.125	PCT.	0.47	1.84	0/ 6	AC02FE	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.546060
112	FAT	MILK	INFR	3.132	PCT.	0.26	2.28	0/ 6	AC23FK	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.676856
113	FAT	MILK	INFR	3.147	PCT.	0.26	1.02	0/ 6	AC02FB	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.303022
114	FAT	MILK	INFR	3.147	PCT.	0.49	2.09	0/ 6	AC23FG	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.620923
115	FAT	MILK	INFR	3.153	PCT.	0.87	1.60	0/ 6	AC02FD	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.475480
116	FAT	MILK	INFR	3.167	PCT.	0.41	2.75	0/ 6	AC26FG	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.817751
117	FAT	MILK	INFR	3.195	PCT.	0.69	2.15	0/ 6	AC20FK	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.640190
118	FAT	MILK	INFR	3.198	PCT.	0.74	2.63	0/ 6	AC20FG	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.783239
119	FAT	MILK	INFR	3.295	PCT.	.	1.74	0/ 4	AM08B	<16.083 84 >	.BIGGS.55,.488.(1972).	0.520516
120	FAT	MILK	INFR	3.296	PCT.	.	0.82	0/ 5	AM03B	<16.083 84 >	.BIGGS.55,.488.(1972).	0.245312
121	FAT	MILK	INFR	3.368	PCT.	1.06	1.28	0/ 6	AC15FJ	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.384179
122	FAT	MILK	INFR	3.385	PCT.	0.54	1.22	0/ 6	AC15FF	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.366443
123	FAT	MILK	INFR	3.408	PCT.	.	0.32	0/ 5	AM01B	<16.083 84 >	.BIGGS.55,.488.(1972).	0.096214
124	FAT	MILK	INFR	3.429	PCT.	0.60	2.25	0/ 6	AC03FI	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.677137
125	FAT	MILK	INFR	3.440	PCT.	0.38	2.01	0/ 6	AC03FC	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.605196
126	FAT	MILK	INFR	3.454	PCT.	0.66	2.19	0/ 6	AC03FE	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.659801
127	FAT	MILK	INFR	3.463	PCT.	0.35	2.25	0/ 6	AC05FC	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.678148
128	FAT	MILK	INFR	3.466	PCT.	0.46	0.91	0/ 6	AC12FF	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.274303
129	FAT	MILK	INFR	3.470	PCT.	.	1.73	0/ 4	AM06B	<16.083 84 >	.BIGGS.55,.488.(1972).	0.521571
130	FAT	MILK	INFR	3.474	PCT.	0.57	2.50	0/ 6	AC05FI	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.753852
131	FAT	MILK	INFR	3.474	PCT.	0.49	1.46	0/ 6	AC12FJ	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.440250
132	FAT	MILK	INFR	3.484	PCT.	0.27	1.91	0/ 6	AC05FE	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.576192
133	FAT	MILK	INFR	3.494	PCT.	0.47	1.73	0/ 6	AC03FH	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.522117
134	FAT	MILK	INFR	3.496	PCT.	0.27	0.95	0/ 6	AC03FB	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.286732
135	FAT	MILK	INFR	3.496	PCT.	1.16	1.25	0/ 6	AC05FH	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.377279
136	FAT	MILK	INFR	3.501	PCT.	0.59	1.83	0/ 6	AC03FD	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.552455
137	FAT	MILK	INFR	3.509	PCT.	0.69	0.98	0/ 6	AC05FD	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.295956
138	FAT	MILK	INFR	3.527	PCT.	0.30	0.78	0/ 6	AC05FB	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.235742
139	FAT	MILK	INFR	3.914	PCT.	0.20	2.40	0/ 6	AC06FI	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.736805
140	FAT	MILK	INFR	3.918	PCT.	0.50	2.37	0/ 6	AC06FC	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.727711
141	FAT	MILK	INFR	3.919	PCT.	0.46	1.95	0/ 6	AC06FE	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.598769
142	FAT	MILK	INFR	3.925	PCT.	1.29	1.31	0/ 6	AC16FJ	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.402340
143	FAT	MILK	INFR	3.940	PCT.	0.83	1.79	0/ 6	AC16FF	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.550078
144	FAT	MILK	INFR	3.972	PCT.	0.64	2.61	0/ 6	AC06FH	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.803061
145	FAT	MILK	INFR	3.992	PCT.	0.22	0.55	0/ 6	AC06FB	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.169355
146	FAT	MILK	INFR	3.992	PCT.	0.54	1.21	0/ 6	AC06FD	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.372582
147	FAT	MILK	INFR	4.077	PCT.	0.83	1.98	0/ 6	AC13FJ	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.611616
148	FAT	MILK	INFR	4.082	PCT.	0.59	1.66	0/ 6	AC13FF	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.512848
149	FAT	MILK	INFR	4.200	PCT.	.	0.17	0/ 5	AM05B	<16.083 84 >	.BIGGS.55,.488.(1972).	0.052747
150	FAT	MILK	INFR	4.247	PCT.	.	1.72	0/ 4	AM09B	<16.083 84 >	.BIGGS.55,.488.(1972).	0.534579
151	FAT	MILK	INFR	4.247	PCT.	.	1.72	0/ 4	AM10B	<16.083 84 >	.BIGGS.55,.488.(1972).	0.534579
152	FAT	MILK	INFR	4.409	PCT.	0.71	2.17	0/ 6	AC17FJ	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.678243
153	FAT	MILK	INFR	4.416	PCT.	0.69	0.96	0/ 6	AC17FF	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.300120
154	FAT	MILK	INFR	4.677	PCT.	0.76	2.93	0/ 6	AC07FI	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.923939
155	FAT	MILK	INFR	4.687	PCT.	0.35	1.86	1/ 6	AC07FE	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.586722
156	FAT	MILK	INFR	4.698	PCT.	0.21	2.53	0/ 6	AC07FC	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.798359
157	FAT	MILK	INFR	4.742	PCT.	0.69	2.45	0/ 6	AC07FH	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.774204
158	FAT	MILK	INFR	4.793	PCT.	0.49	1.90	0/ 6	AC07FD	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.601367
159	FAT	MILK	INFR	4.804	PCT.	0.13	1.50	1/ 6	AC07FB	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.474922
160	FAT	MILK	INFR	4.851	PCT.	0.55	2.34	0/ 6	AC08FI	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.741962
161	FAT	MILK	INFR	4.878	PCT.	0.69	2.27	0/ 6	AC08FE	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.720379
162	FAT	MILK	INFR	4.885	PCT.	0.22	2.62	0/ 6	AC08FC	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.831622
163	FAT	MILK	INFR	4.909	PCT.	1.23	3.09	1/ 6	AC08FH	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.981529
164	FAT	MILK	INFR	4.947	PCT.	0.45	2.08	0/ 6	AC08FD	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.661483
165	FAT	MILK	INFR	4.977	PCT.	0.40	1.37	0/ 6	AC08FB	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.436074
166	FAT	MILK	INFR	5.076	PCT.	.	0.41	0/ 5	AM02B	<16.083 84 >	.BIGGS.55,.488.(1972).	0.130893
167	FAT	MILK	INFR	5.190	PCT.	.	1.03	0/ 4	AM07B	<16.083 84 >	.BIGGS.55,.488.(1972).	0.329929
168	FAT	MILK	VOLU	2.662	PCT.	.	0.41	0/ 5	AM09A	<16.065 84 >	.BIGGS.55,.488.(1972).	0.118775
169	FAT	MILK	VOLU	2.672	PCT.	.	0.77	0/ 4	AM04A	<16.065 84 >	.BIGGS.55,.488.(1972).	0.223197
170	FAT	MILK	VOLU	2.922	PCT.	0.81	1.50	0/ 9	AD07	<16.065 84 >	.MCKINLEY.58,.949.(1975).	0.440685
171	FAT	MILK	VOLU	3.000	PCT.	.	0.00	0/ 6	AL02A	<16.065 84 >	.KING.56,.1401(1973).	0.000000

OBS	ANALYTE	MATRIX	METHOD	CONCN	UNITS	RSDO	RSDR	DROPPED/ # LABS	CODE	METHOD NO.	REFERENCE	HORRAT
172	FAT	MILK	VOLU	3.039	PCT.	0.55	1.23	0/ 9	AD01	<16.065 84 >	.MCKINLEY.58,.949.(1975).	0.363497
173	FAT	MILK	VOLU	3.173	PCT.	.	2.55	0/ 6	AL01A	<16.065 84 >	.KING.56,.1401(1973).	0.758518
174	FAT	MILK	VOLU	3.200	PCT.	0.00	0.00	2/ 9	AD03	<16.065 84 >	.MCKINLEY.58,.949.(1975).	0.000000
175	FAT	MILK	VOLU	3.260	PCT.	2.47	2.47	1/ 6	AN01B	<16.065 84 >	.MITCHELL.50,.537.(1967).	0.737707
176	FAT	MILK	VOLU	3.263	PCT.	1.02	1.91	0/ 6	AN04B	<16.065 84 >	.MITCHELL.50,.537.(1967).	0.570541
177	FAT	MILK	VOLU	3.275	PCT.	.	1.55	0/ 4	AM03A	<16.065 84 >	.BIGGS.55,.488.(1972).	0.463254
178	FAT	MILK	VOLU	3.290	PCT.	.	0.80	0/ 5	AM08A	<16.065 84 >	.BIGGS.55,.488.(1972).	0.239263
179	FAT	MILK	VOLU	3.313	PCT.	0.00	1.07	1/ 9	AD02	<16.065 84 >	.MCKINLEY.58,.949.(1975).	0.320343
180	FAT	MILK	VOLU	3.337	PCT.	.	1.49	0/ 6	AL05A	<16.065 84 >	.KING.56,.1401(1973).	0.446573
181	FAT	MILK	VOLU	3.347	PCT.	.	0.90	0/ 6	AL03A	<16.065 84 >	.KING.56,.1401(1973).	0.269864
182	FAT	MILK	VOLU	3.396	PCT.	.	0.26	1/ 6	AL06A	<16.065 84 >	.KING.56,.1401(1973).	0.078133
183	FAT	MILK	VOLU	3.400	PCT.	.	0.00	0/ 4	AM01A	<16.065 84 >	.BIGGS.55,.488.(1972).	0.000000
184	FAT	MILK	VOLU	3.406	PCT.	.	0.64	0/ 5	AM06A	<16.065 84 >	.BIGGS.55,.488.(1972).	0.192411
185	FAT	MILK	VOLU	3.425	PCT.	0.00	0.78	1/ 9	AD05	<16.065 84 >	.MCKINLEY.58,.949.(1975).	0.234698
186	FAT	MILK	VOLU	3.543	PCT.	1.35	2.25	0/ 6	AN05B	<16.065 84 >	.MITCHELL.50,.537.(1967).	0.680483
187	FAT	MILK	VOLU	3.544	PCT.	0.47	1.40	0/ 9	AD08	<16.065 84 >	.MCKINLEY.58,.949.(1975).	0.423432
188	FAT	MILK	VOLU	3.592	PCT.	0.73	1.22	0/ 9	AD10	<16.065 84 >	.MCKINLEY.58,.949.(1975).	0.369726
189	FAT	MILK	VOLU	3.710	PCT.	.	0.42	0/ 6	AL04A	<16.065 84 >	.KING.56,.1401(1973).	0.127905
190	FAT	MILK	VOLU	3.746	PCT.	1.69	2.62	0/ 6	AN07B	<16.065 84 >	.MITCHELL.50,.537.(1967).	0.799041
191	FAT	MILK	VOLU	3.752	PCT.	2.87	2.87	0/ 6	AN03B	<16.065 84 >	.MITCHELL.50,.537.(1967).	0.875490
192	FAT	MILK	VOLU	3.772	PCT.	.	1.07	0/ 6	AL07A	<16.065 84 >	.KING.56,.1401(1973).	0.326664
193	FAT	MILK	VOLU	3.775	PCT.	0.00	0.71	1/ 9	AD04	<16.065 84 >	.MCKINLEY.58,.949.(1975).	0.216787
194	FAT	MILK	VOLU	3.810	PCT.	.	0.41	0/ 6	AL12A	<16.065 84 >	.KING.56,.1401(1973).	0.125361
195	FAT	MILK	VOLU	3.838	PCT.	.	1.28	0/ 6	AL09A	<16.065 84 >	.KING.56,.1401(1973).	0.391807
196	FAT	MILK	VOLU	3.862	PCT.	1.28	1.97	0/ 6	AN02B	<16.065 84 >	.MITCHELL.50,.537.(1967).	0.603585
197	FAT	MILK	VOLU	3.906	PCT.	0.45	0.65	1/ 9	AD06	<16.065 84 >	.MCKINLEY.58,.949.(1975).	0.199490
198	FAT	MILK	VOLU	4.005	PCT.	.	1.61	0/ 6	AL10A	<16.065 84 >	.KING.56,.1401(1973).	0.495983
199	FAT	MILK	VOLU	4.100	PCT.	0.57	0.95	0/ 9	AD09	<16.065 84 >	.MCKINLEY.58,.949.(1975).	0.293695
200	FAT	MILK	VOLU	4.205	PCT.	.	1.53	0/ 6	AL08A	<16.065 84 >	.KING.56,.1401(1973).	0.474808
201	FAT	MILK	VOLU	4.205	PCT.	.	0.14	0/ 4	AM05A	<16.065 84 >	.BIGGS.55,.488.(1972).	0.043446
202	FAT	MILK	VOLU	4.244	PCT.	.	1.32	0/ 5	AM10A	<16.065 84 >	.BIGGS.55,.488.(1972).	0.410207
203	FAT	MILK	VOLU	4.598	PCT.	.	0.35	0/ 6	AL11A	<16.065 84 >	.KING.56,.1401(1973).	0.110088
204	FAT	MILK	VOLU	4.733	PCT.	.	0.90	0/ 6	AL13A	<16.065 84 >	.KING.56,.1401(1973).	0.284319
205	FAT	MILK	VOLU	4.748	PCT.	.	0.86	0/ 6	AL15A	<16.065 84 >	.KING.56,.1401(1973).	0.271812
206	FAT	MILK	VOLU	5.080	PCT.	.	0.36	0/ 4	AM02A	<16.065 84 >	.BIGGS.55,.488.(1972).	0.114944
207	FAT	MILK	VOLU	5.134	PCT.	.	0.45	0/ 5	AM07A	<16.065 84 >	.BIGGS.55,.488.(1972).	0.143909
208	FAT	MILK	VOLU	5.508	PCT.	.	1.09	0/ 6	AL14A	<16.065 84 >	.KING.56,.1401(1973).	0.352291
209	FAT	MILK-CHO	GRAV	2.025	PCT.	2.10	2.10	0/ 5	AN11A	<16.064 84 >	.MITCHELL.50,.537.(1967).	0.583823
210	FAT	MILK-CHO	GRAV	2.043	PCT.	1.97	2.12	0/ 6	AN10A	<16.064 84 >	.MITCHELL.50,.537.(1967).	0.590183
211	FAT	MILK-CHO	GRAV	3.202	PCT.	0.93	1.41	0/ 6	AN12A	<16.064 84 >	.MITCHELL.50,.537.(1967).	0.419977
212	FAT	MILK-CHO	GRAV	3.223	PCT.	0.37	1.09	0/ 6	AN09A	<16.064 84 >	.MITCHELL.50,.537.(1967).	0.324993
213	FAT	MILK-ORI	GRAV	10.940	PCT.	0.56	1.18	0/ 4	BI03E	<16.219 84 >	.EISSES.IDFBUL235,0042(1988).	0.42287
214	FAT	MILK-ORI	GRAV	10.960	PCT.	0.45	0.88	0/ 4	BI04E	<16.219 84 >	.EISSES.IDFBUL235,0042(1988).	0.31545
215	FAT	MILK-ORI	GRAV	11.020	PCT.	0.67	0.67	0/ 4	BI05E	<16.219 84 >	.EISSES.IDFBUL235,0042(1988).	0.24037
216	FAT	MILK-ORI	GRAV	11.496	PCT.	0.66	1.97	0/ 5	BL02B	<16.219 84 >	.KEISTER.10,0308(1927).	0.71127
217	FAT	MILK-ORI	GRAV	11.620	PCT.	0.64	0.64	0/ 4	BI03D	<16.219 84 >	.EISSES.IDFBUL235,0042(1988).	0.23145
218	FAT	MILK-ORI	GRAV	11.680	PCT.	0.21	0.68	0/ 4	BI05D	<16.219 84 >	.EISSES.IDFBUL235,0042(1988).	0.24610
219	FAT	MILK-ORI	GRAV	11.690	PCT.	0.31	0.57	0/ 4	BI04D	<16.219 84 >	.EISSES.IDFBUL235,0042(1988).	0.20632
220	FAT	MILK-ORI	GRAV	23.940	PCT.	0.32	0.39	0/ 4	BI03A	<16.219 84 >	.EISSES.IDFBUL235,0042(1988).	0.15725
221	FAT	MILK-ORI	GRAV	24.020	PCT.	0.26	0.55	0/ 4	BI05A	<16.219 84 >	.EISSES.IDFBUL235,0042(1988).	0.22187
222	FAT	MILK-ORI	GRAV	24.080	PCT.	0.22	0.25	0/ 4	BI04A	<16.219 84 >	.EISSES.IDFBUL235,0042(1988).	0.10089
223	FAT	MILK-ORI	GRAV	24.360	PCT.	0.31	0.61	0/ 4	BI03C	<16.219 84 >	.EISSES.IDFBUL235,0042(1988).	0.24660
224	FAT	MILK-ORI	GRAV	24.500	PCT.	0.06	0.29	0/ 4	BI05C	<16.219 84 >	.EISSES.IDFBUL235,0042(1988).	0.11734
225	FAT	MILK-ORI	GRAV	24.560	PCT.	0.09	0.24	0/ 4	BI04C	<16.219 84 >	.EISSES.IDFBUL235,0042(1988).	0.09714
226	FAT	MILK-ORI	GRAV	25.480	PCT.	0.18	0.41	0/ 4	BI03B	<16.219 84 >	.EISSES.IDFBUL235,0042(1988).	0.16687
227	FAT	MILK-ORI	GRAV	25.610	PCT.	0.15	0.36	0/ 4	BI05B	<16.219 84 >	.EISSES.IDFBUL235,0042(1988).	0.14663
228	FAT	MILK-ORI	GRAV	25.690	PCT.	0.23	0.38	0/ 4	BI04B	<16.219 84 >	.EISSES.IDFBUL235,0042(1988).	0.15485
229	FAT	MILK-ORI	GRAV	25.700	PCT.	0.67	0.67	0/ 5	BI01E	<16.219 84 >	.EISSES.IDFBUL235,0042(1988).	0.27304
230	FAT	MILK-ORI	GRAV	25.770	PCT.	0.21	0.44	0/ 5	BI02E	<16.219 84 >	.EISSES.IDFBUL235,0042(1988).	0.17939
231	FAT	MILK-ORI	GRAV	26.010	PCT.	0.44	0.56	0/ 5	BI01A	<16.219 84 >	.EISSES.IDFBUL235,0042(1988).	0.22863
232	FAT	MILK-ORI	GRAV	26.040	PCT.	0.07	0.29	0/ 5	BI02A	<16.219 84 >	.EISSES.IDFBUL235,0042(1988).	0.11842
233	FAT	MILK-ORI	GRAV	26.080	PCT.	0.96	0.96	0/ 5	BI01B	<16.219 84 >	.EISSES.IDFBUL235,0042(1988).	0.39209
234	FAT	MILK-ORI	GRAV	26.190	PCT.	0.36	0.54	0/ 5	BI02B	<16.219 84 >	.EISSES.IDFBUL235,0042(1988).	0.22069
235	FAT	MILK-ORI	GRAV	26.210	PCT.	0.28	0.65	0/ 5	BI01C	<16.219 84 >	.EISSES.IDFBUL235,0042(1988).	0.26568
236	FAT	MILK-ORI	GRAV	26.290	PCT.	0.40	0.75	0/ 5	BI02C	<16.219 84 >	.EISSES.IDFBUL235,0042(1988).	0.30669
237	FAT	MILK-ORI	GRAV	26.698	PCT.	0.21	2.24	0/ 5	BL01B	<16.219 84 >	.KEISTER.10,0308(1927).	0.91811
238	FAT	MILK-ORI	GRAV	27.135	PCT.	0.46	1.80	0/ 7	AZ01A1	<16.219A 84	.TREUTHARDT.15,.524.(1932).	0.73957
239	FAT	MILK-ORI	GRAV	27.146	PCT.	0.89	2.08	0/ 7	AZ01B1	<16.219B 84	.TREUTHARDT.15,.524.(1932).	0.85467
240	FAT	MILK-ORI	GRAV	27.836	PCT.	0.77	2.26	0/ 7	AZ01B2	<16.219B 84	.TREUTHARDT.15,.524.(1932).	0.93214
241	FAT	MILK-ORI	GRAV	27.860	PCT.	0.90	0.98	0/ 5	BI01D	<16.219 84 >	.EISSES.IDFBUL235,0042(1988).	0.40426
242	FAT	MILK-ORI	GRAV	27.890	PCT.	0.46	1.86	0/ 7	AZ01A2	<16.219A 84	.TREUTHARDT.15,.524.(1932).	0.76739
243	FAT	MILK-ORI	GRAV	27.997	PCT.	0.45	1.66	0/ 7	AZ02A1	<16.219A 84	.TREUTHARDT.15,.524.(1932).	0.68527
244	FAT	MILK-ORI	GRAV	28.033	PCT.	0.43	1.67	0/ 7	AZ02B1	<16.219B 84	.TREUTHARDT.15,.524.(1932).	0.68953
245	FAT	MILK-ORI	GRAV	28.160	PCT.	0.20	0.54	0/ 5	BI02D	<16.219 84 >	.EISSES.IDFBUL235,0042(1988).	0.22311
246	FAT	MILK-ORI	GRAV	28.593	PCT.	0.45	1.53	0/ 7	AZ02A2	<16.219A 84	.TREUTHARDT.15,.524.(1932).	0.63361
247	FAT	MILK-ORI	GRAV	28.632	PCT.	0.42	1.62	0/ 7	AZ02B2	<16.219B 84	.TREUTHARDT.15,.524.(1932).	0.67102
248	FAT-DRY-BASIS	CHEESE-C	GRAV	50.327	PCT.	0.64	1.29	0/ 5	AV03F	<16.284 84 >	.HORWITZ.31,.300.(1948).	0.58167
249	FAT-DRY-BASIS	CHEESE-C	GRAV	50.571	PCT.	1.07	1.32	0/ 5	AV01E	<16.284 84 >	.HORWITZ.31,.300.(1948).	0.59563
250	FAT-DRY-BASIS	CHEESE-C	GRAV	50.571	PCT.	0.94	1.08	0/ 5	AV03E	<16.284 84 >	.HORWITZ.31,.300.(1948).	0.48733
251	FAT-DRY-BASIS	CHEESE-C	GRAV	50.612	PCT.	0.42	1.53	0/ 5	AV01F	<16.284 84 >	.HORWITZ.31,.300.(1948).	0.69047
252	FAT-DRY-BASIS	CHEESE-C	GRAV	54.581	PCT.	0.35	1.32	0/ 5	AV02F	<16.284 84 >	.HORWITZ.31,.300.(1948).	0.60251
253	FAT-DRY-BASIS	CHEESE-C	GRAV	54.722	PCT.	0.52	1.38	0/ 5	AV02E	<16.284 84 >	.HORWITZ.31,.300.(1948).	0.63014
254	FREEZING-POINT	MILK	PHYS	0.424		.	1.06	0/19	AH1A	<16.096 84 >	.HENNINGSON.51,816.(1968).	.
255	FREEZING-POINT	MILK	PHYS	0.623		.	0.64	0/19	AH1B	<16.096 84 >	.HENNINGSON.51,816.(1968).	.
256	FREEZING-POINT	MILK	PHYS	0.432		.	0.77	1/19	AH2A	<16.096 84 >	.HENNINGSON.51,816.(1968).	.
257	FREEZING-POINT	MILK	PHYS	0.630		.	0.93	0/19	AH2B	<16.096 84 >	.HENNINGSON.51,816.(1968).	.
258	FREEZING-POINT	MILK	PHYS	0.518		.	0.86	0/19	AH3A	<16.096 84 >	.HENNINGSON.51,816.(1968).	.
259	FREEZING-POINT	MILK	PHYS	0.512		.	0.71	1/19	AH3B	<16.096 84 >	.HENNINGSON.51,816.(1968).	.
260	FREEZING-POINT	MILK	PHYS	0.512		.	0.71	1/19	AH4A	<16.096 84 >	.HENNINGSON.51,816.(1968).	.
261	LACTOSE	MILK	ENZY	3.617	PCT.	2.99	5.34	0/ 8	BG04A	<16.059 84 >	.KLEYN.67,.0637(1984).	1.62006
262	LACTOSE	MILK	ENZY	3.859	PCT.	.	3.14	0/ 8	BG06A	<16.059 84 >	.KLEYN.67,.0637(1984).	0.96192
263	LACTOSE	MILK	ENZY	3.862	PCT.	.	5.11	0/ 8	BG07A	<16.059 84 >	.KLEYN.67,.0637(1984).	1.56565
264	LACTOSE	MILK	ENZY	4.112	PCT.	4.20	4.85	1/ 8	BG01A	<16.059 84 >	.KLEYN.67,.0637(1984).	1.50006
265	LACTOSE	MILK	ENZY	4.179	PCT.	.	4.78	1/ 8	BG05A	<16.059 84 >	.KLEYN.67,.0637(1984).	1.48198
266	LACTOSE	MILK	ENZY	4.286	PCT.	.	6.52	0/ 8	BG02A	<16.059 84 >	.KLEYN.67,.0637(1984).	2.02920
267	LACTOSE	MILK	ENZY	4.496	PCT.	3.43	5.67	1/ 8	BG03A	<16.059 84 >	.KLEYN.67,.0637(1984).	1.77742
268	LACTOSE	MILK	INFR	4.602	PCT.	.	2.14	0/ 5	AM02F	<16.083 84 >	.BIGGS.55,.488.(1972).	0.67319
269	LACTOSE	MILK	INFR	4.644	PCT.	.	2.03	0/ 5	AM04F	<16.083 84 >	.BIGGS.55,.488.(1972).	0.63946
270	LACTOSE	MILK	INFR	4.644	PCT.	.	2.03	0/ 5	AM05F	<16.083 84 >	.BIGGS.55,.488.(1972).	0.63946
271	LACTOSE	MILK	INFR	4.654	PCT.	.	2.23	0/ 5	AM01F	<16.083 84 >	.BIGGS.55,.488.(1972).	0.70269
272	LACTOSE	MILK	INFR	4.666	PCT.	.	2.19	0/ 5	AM03F	<16.083 84 >	.BIGGS.55,.488.(1972).	0.69035
273	LACTOSE	MILK	INFR	4.670	PCT.	.	3.27	0/ 4	AM09F	<16.083 84 >	.BIGGS.55,.488.(1972).	1.03093
274	LACTOSE	MILK	INFR	4.720	PCT.	.	3.42	0/ 4	AM06F	<16.083 84 >	.BIGGS.55,.488.(1972).	1.07995
275	LACTOSE	MILK	INFR	4.750	PCT.	.	4.07	0/ 4	AM07F	<16.083 84 >	.BIGGS.55,.488.(1972).	1.28643
276	LACTOSE	MILK	INFR	4.750	PCT.	.	3.32	0/ 4	AM08F	<16.083 84 >	.BIGGS.55,.488.(1972).	1.04937
277	LACTOSE	MILK	INFR	4.800	PCT.	.	3.61	0/ 4	AM10F	<16.083 84 >	.BIGGS.55,.488.(1972).	1.14284
278	LACTOSE	MILK	POLI	4.530	PCT.	.	1.56	0/ 2	AM04E	<16.055 84 >	.BIGGS.55,.488.(1972).	0.48957
279	LACTOSE	MILK	POLI	4.530	PCT.	.	1.23	0/ 3	AM09E	<16.055 84 >	.BIGGS.55,.488.(1972).	0.38601
280	LACTOSE	MILK	POLI	4.600	PCT.	.	1.09	0/ 3	AM06E	<16.055 84 >	.BIGGS.55,.488.(1972).	0.34286
281	LACTOSE	MILK	POLI	4.615	PCT.	.	1.38	0/ 2	AM01E	<16.055 84 >	.BIGGS.55,.488.(1972).	0.43430
282	LACTOSE	MILK	POLI	4.620	PCT.	.	1.84	0/ 2	AM02E	<16.055 84 >	.BIGGS.55,.488.(1972).	0.57916
283	LACTOSE	MILK	POLI	4.635	PCT.	.	1.68	0/ 2	AM03E	<16.055 84 >	.BIGGS.55,.488.(1972).	0.52905
284	LACTOSE	MILK	POLI	4.637	PCT.	.	1.11	0/ 3	AM07E	<16.055 84 >	.BIGGS.55,.488.(1972).	0.34957
285	LACTOSE	MILK	POLI	4.650	PCT.	.	1.82	0/ 2	AM05E	<16.055 84 >	.BIGGS.55,.488.(1972).	0.57342
286	LACTOSE	MILK	POLI	4.653	PCT.	.	1.02	0/ 3	AM08E	<16.055 84 >	.BIGGS.55,.488.(1972).	0.32140
287	LACTOSE	MILK	POLI	4.667	PCT.	.	1.01	0/ 3	AM10E	<16.055 84 >	.BIGGS.55,.488.(1972).	0.31839
288	MOISTURE	BUTTER	GRAV	16.208	PCT.	0.19	2.24	0/ 6	BD6A	<16.231 84 >	.WEBER.35,.194.(1952).	0.85166
289	MOISTURE	BUTTER	GRAV	16.291	PCT.	0.14	1.11	0/ 6	BD1A	<16.231 84 >	.WEBER.35,.194.(1952).	0.42236

OBS	ANALYTE	MATRIX	METHOD	CONCN	UNITS	RSDO	RSDR	DROPPED/ # LABS	CODE	METHOD NO.	REFERENCE	HORRAT
290	MOISTURE	BUTTER	GRAV	16.327	PCT.	0.13	0.69	1/ 6	BD5A	<16.231 84 >	.WEBER.35,.194.(1952).	0.26263
291	MOISTURE	BUTTER	GRAV	16.336	PCT.	0.49	1.59	0/ 6	BD3A	<16.231 84 >	.WEBER.35,.194.(1952).	0.60525
292	MOISTURE	BUTTER	GRAV	16.346	PCT.	0.19	1.46	1/ 6	BD4A	<16.231 84 >	.WEBER.35,.194.(1952).	0.55581
293	MOISTURE	BUTTER	GRAV	16.431	PCT.	0.18	1.47	0/ 6	BD2A	<16.231 84 >	.WEBER.35,.194.(1952).	0.56006
294	MOISTURE	CHEESE	GRAV	37.730	PCT.	0.32	0.67	1/ 6	AE16B	<16.259 84 >	.PIEPER.60,.1392.(1977).	0.28929
295	MOISTURE	CHEESE	GRAV	37.742	PCT.	0.20	0.58	0/ 6	AE16A	<16.265 84 >	.PIEPER.60,.1392.(1977).	0.25044
296	MOISTURE	CHEESE	GRAV	37.767	PCT.	0.19	0.84	0/ 6	AE2A	<16.265 84 >	.PIEPER.60,.1392.(1977).	0.36274
297	MOISTURE	CHEESE	GRAV	37.767	PCT.	0.29	0.76	0/ 6	AE2B	<16.259 84 >	.PIEPER.60,.1392.(1977).	0.32820
298	MOISTURE	CHEESE	GRAV	37.790	PCT.	0.14	0.15	0/ 5	AE4A	<16.265 84 >	.PIEPER.60,.1392.(1977).	0.06478
299	MOISTURE	CHEESE	GRAV	37.830	PCT.	0.19	0.45	0/ 5	AE4B	<16.259 84 >	.PIEPER.60,.1392.(1977).	0.19438
300	MOISTURE	CHEESE	GRAV	37.925	PCT.	0.41	0.91	0/ 6	AE1B	<16.259 84 >	.PIEPER.60,.1392.(1977).	0.39322
301	MOISTURE	CHEESE	GRAV	38.008	PCT.	0.20	0.61	0/ 6	AE1A	<16.265 84 >	.PIEPER.60,.1392.(1977).	0.26367
302	MOISTURE	CHEESE	GRAV	38.342	PCT.	0.31	0.35	0/ 6	AE6A	<16.265 84 >	.PIEPER.60,.1392.(1977).	0.15149
303	MOISTURE	CHEESE	GRAV	38.350	PCT.	0.37	0.69	0/ 6	AE6B	<16.259 84 >	.PIEPER.60,.1392.(1977).	0.29865
304	MOISTURE	CHEESE	GRAV	38.625	PCT.	0.22	0.71	0/ 6	AE5A	<16.265 84 >	.PIEPER.60,.1392.(1977).	0.30764
305	MOISTURE	CHEESE	GRAV	38.642	PCT.	0.33	0.92	0/ 6	AE5B	<16.259 84 >	.PIEPER.60,.1392.(1977).	0.39866
306	MOISTURE	CHEESE	GRAV	38.953	PCT.	0.49	0.67	0/ 6	AE3A	<16.265 84 >	.PIEPER.60,.1392.(1977).	0.29068
307	MOISTURE	CHEESE	GRAV	38.967	PCT.	0.28	0.63	0/ 6	AE3B	<16.259 84 >	.PIEPER.60,.1392.(1977).	0.27334
308	MOISTURE	CHEESE	GRAV	39.533	PCT.	0.23	0.44	0/ 6	AE10B	<16.259 84 >	.PIEPER.60,.1392.(1977).	0.19132
309	MOISTURE	CHEESE	GRAV	39.650	PCT.	0.23	0.53	0/ 6	AE10A	<16.265 84 >	.PIEPER.60,.1392.(1977).	0.23056
310	MOISTURE	CHEESE	GRAV	39.733	PCT.	0.21	1.26	0/ 6	AE11B	<16.259 84 >	.PIEPER.60,.1392.(1977).	0.54829
311	MOISTURE	CHEESE	GRAV	39.908	PCT.	0.26	1.22	0/ 6	AE11A	<16.265 84 >	.PIEPER.60,.1392.(1977).	0.53123
312	MOISTURE	CHEESE	GRAV	40.180	PCT.	0.00	0.54	1/ 6	AE7A	<16.265 84 >	.PIEPER.60,.1392.(1977).	0.23538
313	MOISTURE	CHEESE	GRAV	40.225	PCT.	0.40	0.98	0/ 6	AE7B	<16.259 84 >	.PIEPER.60,.1392.(1977).	0.42723
314	MOISTURE	CHEESE	GRAV	40.425	PCT.	0.40	1.29	0/ 6	AE13B	<16.259 84 >	.PIEPER.60,.1392.(1977).	0.56280
315	MOISTURE	CHEESE	GRAV	40.458	PCT.	0.34	0.70	0/ 6	AE9B	<16.259 84 >	.PIEPER.60,.1392.(1977).	0.30543
316	MOISTURE	CHEESE	GRAV	40.583	PCT.	0.14	0.86	0/ 6	AE9A	<16.265 84 >	.PIEPER.60,.1392.(1977).	0.37542
317	MOISTURE	CHEESE	GRAV	40.633	PCT.	0.17	1.02	0/ 6	AE13A	<16.265 84 >	.PIEPER.60,.1392.(1977).	0.44535
318	MOISTURE	CHEESE	GRAV	40.783	PCT.	0.22	0.70	0/ 6	AE12B	<16.259 84 >	.PIEPER.60,.1392.(1977).	0.30580
319	MOISTURE	CHEESE	GRAV	40.858	PCT.	0.23	0.80	0/ 6	AE12A	<16.265 84 >	.PIEPER.60,.1392.(1977).	0.34958
320	MOISTURE	CHEESE	GRAV	41.017	PCT.	0.28	0.49	0/ 6	AE8A	<16.265 84 >	.PIEPER.60,.1392.(1977).	0.21425
321	MOISTURE	CHEESE	GRAV	41.025	PCT.	0.27	0.68	0/ 6	AE8B	<16.259 84 >	.PIEPER.60,.1392.(1977).	0.29733
322	MOISTURE	CHEESE	GRAV	41.508	PCT.	0.18	0.65	0/ 6	AE14A	<16.265 84 >	.PIEPER.60,.1392.(1977).	0.28471
323	MOISTURE	CHEESE	GRAV	41.667	PCT.	0.24	0.51	0/ 6	AE14B	<16.259 84 >	.PIEPER.60,.1392.(1977).	0.22352
324	MOISTURE	CHEESE	GRAV	41.837	PCT.	0.33	1.01	0/ 6	AE15A	<16.265 84 >	.PIEPER.60,.1392.(1977).	0.44292
325	MOISTURE	CHEESE	GRAV	41.850	PCT.	0.10	0.96	0/ 6	AE15B	<16.259 84 >	.PIEPER.60,.1392.(1977).	0.42102
326	MOISTURE	CHEESE	GRAV	42.833	PCT.	0.29	0.86	0/ 6	AE17B	<16.259 84 >	.PIEPER.60,.1392.(1977).	0.37848
327	MOISTURE	CHEESE	GRAV	42.958	PCT.	0.22	0.62	0/ 6	AE17A	<16.265 84 >	.PIEPER.60,.1392.(1977).	0.27298
328	MOISTURE	CHEESE	GRAV	43.583	PCT.	0.31	0.38	0/ 6	AE18B	<16.259 84 >	.PIEPER.60,.1392.(1977).	0.16767
329	MOISTURE	CHEESE	GRAV	43.642	PCT.	0.26	0.64	0/ 6	AE18A	<16.265 84 >	.PIEPER.60,.1392.(1977).	0.28246
330	MOISTURE	CHEESE-B	GRAV	39.805	PCT.	0.17	0.53	1/12	BE02AV	<16.259 84 >	.STRANGE.52,..117.(1969).	0.23069
331	MOISTURE	CHEESE-B	VOLU	38.846	PCT.	0.38	1.77	0/12	BE03AD	<16.261 84 >	.STRANGE.52,..117.(1969).	0.76760
332	MOISTURE	CHEESE-B	VOLU	39.038	PCT.	0.46	1.19	1/11	BE03BD	<16.261 84 >	.STRANGE.52,..117.(1969).	0.51645
333	MOISTURE	CHEESE-B	VOLU	39.782	PCT.	0.36	0.60	1/12	BE02AD	<16.261 84 >	.STRANGE.52,..117.(1969).	0.26114
334	MOISTURE	CHEESE-C	GRAV	36.008	PCT.	0.30	0.90	0/ 5	AV02A	<16.259 84 >	.HCRWITZ.31,..300.(1948).	0.38587
335	MOISTURE	CHEESE-C	GRAV	36.176	PCT.	0.57	1.01	0/ 5	AV02B	<16.260 84 >	.HCRWITZ.31,..300.(1948).	0.43334
336	MOISTURE	CHEESE-C	GRAV	36.626	PCT.	0.31	0.58	1/ 5	AV03A	<16.259 84 >	.HCRWITZ.31,..300.(1948).	0.24931
337	MOISTURE	CHEESE-C	GRAV	36.632	PCT.	0.81	1.05	0/ 5	AV03B	<16.260 84 >	.HCRWITZ.31,..300.(1948).	0.45135
338	MOISTURE	CHEESE-C	GRAV	39.030	PCT.	0.34	0.35	0/ 5	AV01A	<16.259 84 >	.HCRWITZ.31,..300.(1948).	0.15189
339	MOISTURE	CHEESE-C	GRAV	39.051	PCT.	0.38	0.49	0/ 5	AV01B	<16.260 84 >	.HCRWITZ.31,..300.(1948).	0.21267
340	MOISTURE	CHEESE-C	GRAV	39.225	PCT.	0.27	0.41	2/12	BE01AV	<16.259 84 >	.STRANGE.52,..117.(1969).	0.17807
341	MOISTURE	CHEESE-C	GRAV	39.239	PCT.	0.27	0.57	1/11	BE01BV	<16.259 84 >	.STRANGE.52,..117.(1969).	0.24757
342	MOISTURE	CHEESE-C	VOLU	38.830	PCT.	0.27	1.51	0/12	BE01AD	<16.261 84 >	.STRANGE.52,..117.(1969).	0.65480
343	MOISTURE	CHEESE-C	VOLU	38.950	PCT.	0.60	1.15	0/11	BE01BD	<16.261 84 >	.STRANGE.52,..117.(1969).	0.49892
344	MOISTURE	CHEESE-R	VOLU	43.742	PCT.	0.79	1.47	0/12	BE04AD	<16.261 84 >	.STRANGE.52,..117.(1969).	0.64899
345	MOISTURE	CHEESE-S	GRAV	36.194	PCT.	0.15	0.73	0/11	BC01C	<16.259 84 >	.LEPPER.16,.584.(1933).	0.31323
346	MOISTURE	MILK-DRI	GRAV	2.602	PCT.	1.54	4.24	0/ 5	BL01A	<16.212 84 >	.KEISTER.10,0308(1927).	1.22410
347	MOISTURE	MILK-DRI	GRAV	4.741	PCT.	1.02	1.40	0/ 5	BL02A	<16.212 84 >	.KEISTER.10,0308(1927).	0.44238
348	MOISTURE	MILK-DRI	GRAV	8.371	PCT.	0.50	1.59	0/ 5	BL03A	<16.212 84 >	.KEISTER.10,0308(1927).	0.54730
349	PHOSPHORUS	CHEESE	COLO	0.748	PCT.	1.02	2.41	0/10	AS1A	<IDF33B:1982	.IDFBUL207.4,98(1986).	0.57676
350	PHOSPHORUS	CHEESE	COLO	0.750	PCT.	1.44	2.15	0/10	AS1B	<IDF33B:1982	.IDFBUL207.4,98(1986).	0.51474
351	PHOSPHORUS	CHEESE	COLO	1.083	PCT.	1.25	2.71	0/10	AS3A	<IDF33B:1982	.IDFBUL207.4,98(1986).	0.68570
352	PHOSPHORUS	CHEESE	COLO	1.083	PCT.	1.42	2.72	0/10	AS2B	<IDF33B:1982	.IDFBUL207.4,98(1986).	0.68823
353	PHOSPHORUS	CHEESE	COLO	1.086	PCT.	0.88	2.12	1/10	AS3B	<IDF33B:1982	.IDFBUL207.4,98(1986).	0.53664
354	PHOSPHORUS	CHEESE	COLO	1.089	PCT.	1.76	3.15	0/10	AS2A	<IDF33B:1982	.IDFBUL207.4,98(1986).	0.79773
355	PHOSPHORUS	CHEESE	COLO	1.171	PCT.	0.90	2.74	0/10	AS5B	<IDF33B:1982	.IDFBUL207.4,98(1986).	0.70149
356	PHOSPHORUS	CHEESE	COLO	1.175	PCT.	0.89	1.53	0/10	AS5A	<IDF33B:1982	.IDFBUL207.4,98(1986).	0.39192
357	PHOSPHORUS	CHEESE	COLO	1.477	PCT.	1.62	2.10	0/10	AS7A	<IDF33B:1982	.IDFBUL207.4,98(1986).	0.55672
358	PHOSPHORUS	CHEESE	COLO	1.482	PCT.	3.12	3.74	0/10	AS7B	<IDF33B:1982	.IDFBUL207.4,98(1986).	0.99200
359	PHOSPHORUS	CHEESE	COLO	1.686	PCT.	1.60	3.05	1/10	AS4A	<IDF33B:1982	.IDFBUL207.4,98(1986).	0.82487
360	PHOSPHORUS	CHEESE	COLO	1.698	PCT.	0.77	2.05	1/10	AS4B	<IDF33B:1982	.IDFBUL207.4,98(1986).	0.55502
361	PHOSPHORUS	CHEESE	COLO	1.763	PCT.	1.06	3.46	1/10	AS6B	<IDF33B:1982	.IDFBUL207.4,98(1986).	0.94206
362	PHOSPHORUS	CHEESE	COLO	1.770	PCT.	1.27	4.16	0/10	AS6A	<IDF33B:1982	.IDFBUL207.4,98(1986).	1.13333
363	PHOSPHORUS	CHEESE-P	SPEC	0.823	PCT.	2.05	5.74	1/10	BM08A	<IDF.33A:197	.STEIGER.68,..102.(1985).	1.39362
364	PHOSPHORUS	CHEESE-P	SPEC	0.839	PCT.	2.05	7.16	0/10	BM09A	<IDF.33A:197	.STEIGER.68,..102.(1985).	1.74348
365	PHOSPHORUS	CHEESE-P	SPEC	0.861	PCT.	1.32	4.16	1/10	BM10A	<IDF.33A:197	.STEIGER.68,..102.(1985).	1.01676
366	PHOSPHORUS	CHEESE-P	SPEC	0.873	PCT.	1.74	4.51	1/10	BM02A	<IDF.33A:197	.STEIGER.68,..102.(1985).	1.10464
367	PHOSPHORUS	CHEESE-P	SPEC	0.877	PCT.	1.92	5.71	0/10	BM04A	<IDF.33A:197	.STEIGER.68,..102.(1985).	1.39952
368	PHOSPHORUS	CHEESE-P	SPEC	0.889	PCT.	1.26	6.57	1/10	BM05A	<IDF.33A:197	.STEIGER.68,..102.(1985).	1.61379
369	PHOSPHORUS	CHEESE-P	SPEC	0.927	PCT.	1.82	6.29	0/10	BM03A	<IDF.33A:197	.STEIGER.68,..102.(1985).	1.55460
370	PHOSPHORUS	CHEESE-P	SPEC	0.932	PCT.	1.68	6.89	0/10	BM01A	<IDF.33A:197	.STEIGER.68,..102.(1985).	1.70441
371	PHOSPHORUS	CHEESE-P	SPEC	0.995	PCT.	1.49	3.80	1/10	BM06A	<IDF.33A:197	.STEIGER.68,..102.(1985).	0.94924
372	PHOSPHORUS	CHEESE-P	SPEC	0.999	PCT.	1.92	4.97	1/10	BM07A	<IDF.33A:197	.STEIGER.68,..102.(1985).	1.24224
373	PHOSPHORUS	CHEESE-P	SPEC	1.025	PCT.	2.58	6.75	1/10	BM11A	<IDF.33A:197	.STEIGER.68,..102.(1985).	1.69378
374	PHOSPHORUS	CHEESE-P	SPEC	1.043	PCT.	1.24	5.62	1/10	BM12A	<IDF.33A:197	.STEIGER.68,..102.(1985).	1.41394
375	PHOSPHORUS	MILK	COLO	0.081	PCT.	1.31	9.00	0/ 7	AR2B	<IDF42:1967>	.IDFBUL207.3,41(1986).	1.54255
376	PHOSPHORUS	MILK	COLO	0.082	PCT.	1.80	7.74	0/ 7	AR10B	<IDF42:1967>	.IDFBUL207.3,41(1986).	1.32920
377	PHOSPHORUS	MILK	COLO	0.084	PCT.	0.84	4.01	1/ 7	AR2A	<IDF42:1967>	.IDFBUL207.3,41(1986).	0.69051
378	PHOSPHORUS	MILK	COLO	0.086	PCT.	2.32	2.59	1/ 7	AR1B	<IDF42:1967>	.IDFBUL207.3,41(1986).	0.44771
379	PHOSPHORUS	MILK	COLO	0.087	PCT.	1.90	7.88	0/ 7	AR14B	<IDF42:1967>	.IDFBUL207.3,41(1986).	1.36343
380	PHOSPHORUS	MILK	COLO	0.087	PCT.	1.28	3.12	0/ 6	AR8B	<IDF42:1967>	.IDFBUL207.3,41(1986).	0.54018
381	PHOSPHORUS	MILK	COLO	0.088	PCT.	1.01	3.82	0/ 7	AR1A	<IDF42:1967>	.IDFBUL207.3,41(1986).	0.66250
382	PHOSPHORUS	MILK	COLO	0.089	PCT.	1.13	7.54	1/ 7	AR5B	<IDF42:1967>	.IDFBUL207.3,41(1986).	1.30935
383	PHOSPHORUS	MILK	COLO	0.089	PCT.	1.24	7.62	0/ 7	AR9B	<IDF42:1967>	.IDFBUL207.3,41(1986).	1.32410
384	PHOSPHORUS	MILK	COLO	0.089	PCT.	1.77	5.37	0/ 7	AR10A	<IDF42:1967>	.IDFBUL207.3,41(1986).	0.93357
385	PHOSPHORUS	MILK	COLO	0.090	PCT.	1.65	7.82	0/ 7	AR13B	<IDF42:1967>	.IDFBUL207.3,41(1986).	1.36048
386	PHOSPHORUS	MILK	COLO	0.092	PCT.	1.20	3.81	0/ 7	AR8A	<IDF42:1967>	.IDFBUL207.3,41(1986).	0.66473
387	PHOSPHORUS	MILK	COLO	0.092	PCT.	0.71	8.96	0/ 7	AR16B	<IDF42:1967>	.IDFBUL207.3,41(1986).	1.56380
388	PHOSPHORUS	MILK	COLO	0.092	PCT.	0.77	4.31	1/ 7	AR12B	<IDF42:1967>	.IDFBUL207.3,41(1986).	0.75241
389	PHOSPHORUS	MILK	COLO	0.092	PCT.	2.24	2.24	0/ 6	AR7B	<IDF42:1967>	.IDFBUL207.3,41(1986).	0.39109
390	PHOSPHORUS	MILK	COLO	0.093	PCT.	1.93	4.06	0/ 7	AR14A	<IDF42:1967>	.IDFBUL207.3,41(1986).	0.71000
391	PHOSPHORUS	MILK	COLO	0.094	PCT.	1.87	3.74	0/ 7	AR12A	<IDF42:1967>	.IDFBUL207.3,41(1986).	0.65479
392	PHOSPHORUS	MILK	COLO	0.094	PCT.	5.73	5.73	1/ 7	AR5B	<IDF42:1967>	.IDFBUL207.3,41(1986).	1.00341
393	PHOSPHORUS	MILK	COLO	0.094	PCT.	0.61	3.19	1/ 7	AR6A	<IDF42:1967>	.IDFBUL207.3,41(1986).	0.55869
394	PHOSPHORUS	MILK	COLO	0.094	PCT.	1.86	4.69	0/ 7	AR11B	<IDF42:1967>	.IDFBUL207.3,41(1986).	0.82149
395	PHOSPHORUS	MILK	COLO	0.094	PCT.	2.39	8.47	0/ 7	AR15B	<IDF42:1967>	.IDFBUL207.3,41(1986).	1.48427
396	PHOSPHORUS	MILK	COLO	0.095	PCT.	0.96	2.75	1/ 7	AR7A	<IDF42:1967>	.IDFBUL207.3,41(1986).	0.48214
397	PHOSPHORUS	MILK	COLO	0.095	PCT.	1.78	3.95	0/ 7	AR13A	<IDF42:1967>	.IDFBUL207.3,41(1986).	0.69258
398	PHOSPHORUS	MILK	COLO	0.095	PCT.	0.74	3.73	1/ 7	AR11A	<IDF42:1967>	.IDFBUL207.3,41(1986).	0.65465
399	PHOSPHORUS	MILK	COLO	0.095	PCT.	1.75	4.67	0/ 7	AR9A	<IDF42:1967>	.IDFBUL207.3,41(1986).	0.81985
400	PHOSPHORUS	MILK	COLO	0.097	PCT.	0.92	8.88	0/ 7	AR4B	<IDF42:1967>	.IDFBUL207.3,41(1986).	1.56208
401	PHOSPHORUS	MILK	COLO	0.097	PCT.	0.59	2.52	1/ 7	AR5A	<IDF42:1967>	.IDFBUL207.3,41(1986).	0.44367
402	PHOSPHORUS	MILK	COLO	0.098	PCT.	2.06	6.37	0/ 7	AR16A	<IDF42:1967>	.IDFBUL207.3,41(1986).	1.12228
403	PHOSPHORUS	MILK	COLO	0.098	PCT.	0.98	9.51	0/ 7	AR3B	<IDF42:1967>	.IDFBUL207.3,41(1986).	1.67696
404	PHOSPHORUS	MILK	COLO	0.099	PCT.	1.50	5.57	0/ 7	AR15A	<IDF42:1967>	.IDFBUL207.3,41(1986).	0.98348
405	PHOSPHORUS	MILK	COLO	0.100	PCT.	1.81	3.09	0/ 7	AR4A	<IDF42:1967>	.IDFBUL207.3,41(1986).	0.54647
406	PHOSPHORUS	MILK	COLO	0.102	PCT.	1.06	2.67	1/ 7	AR3A	<IDF42:1967>	.IDFBUL207.3,41(1986).	0.47317
407	POTASSIUM	MILK-DRI	ATAB	1.206	PCT.	1.95	4.65	0/ 7	AT01B	<IDF119:1984	6, 1985 IDFBUL207 6,133(1986)	1.19578

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408	POTASSIUM	MILK-DRI	ATAB	1.279	PCT.	3.85	5.47	0/ 7	AT02B	<IDF119:1984	6, 1985 IDFBUL207 6,133(1986)	1.41911
409	POTASSIUM	MILK-DRI	ATAB	1.540	PCT.	1.59	3.82	0/ 7	AT03B	<IDF119:1984	6, 1985 IDFBUL207 6,133(1986)	1.01917
410	POTASSIUM	MILK-DRI	ATAB	1.547	PCT.	1.59	3.89	0/ 7	AT04B	<IDF119:1984	6, 1985 IDFBUL207 6,133(1986)	1.03850
411	POTASSIUM	MILK-DRI	ATAB	1.670	PCT.	2.36	4.26	0/ 7	AT05B	<IDF119:1984	6, 1985 IDFBUL207 6,133(1986)	1.15048
412	POTASSIUM	MILK-DRI	ATAB	1.693	PCT.	0.49	1.08	1/ 7	AT06B	<IDF119:1984	6, 1985 IDFBUL207 6,133(1986)	0.29227
413	PROTEIN	BUTTER-M	COLO	2.612	PCT.	.	0.64	0/ 7	AF17	<16.037 84 >	.SHERBON.51,.811.(1968).	0.18488
414	PROTEIN	BUTTER-M	COLO	2.670	PCT.	.	0.86	0/ 7	AF15	<16.037 84 >	.SHERBON.51,.811.(1968).	0.24925
415	PROTEIN	BUTTER-M	COLO	2.849	PCT.	.	0.49	0/ 7	AF20	<16.037 84 >	.SHERBON.51,.811.(1968).	0.14341
416	PROTEIN	BUTTER-M	COLO	2.990	PCT.	.	1.05	0/ 7	AF13	<16.037 84 >	.SHERBON.51,.811.(1968).	0.30955
417	PROTEIN	BUTTER-M	COLO	3.065	PCT.	.	1.07	0/ 5	AJ08A	<16.037 84 >	.SHERBON.58,.773.(1975).	0.31662
418	PROTEIN	BUTTER-M	COLO	3.100	PCT.	.	0.71	0/ 7	AF18	<16.037 84 >	.SHERBON.51,.811.(1968).	0.21045
419	PROTEIN	BUTTER-M	COLO	3.120	PCT.	.	0.89	0/ 7	AF14	<16.037 84 >	.SHERBON.51,.811.(1968).	0.26406
420	PROTEIN	BUTTER-M	COLO	3.148	PCT.	.	1.36	0/ 5	AJ08B	<16.037 84 >	.SHERBON.58,.773.(1975).	0.40405
421	PROTEIN	BUTTER-M	COLO	3.231	PCT.	.	0.49	0/ 7	AF16	<16.037 84 >	.SHERBON.51,.811.(1968).	0.14615
422	PROTEIN	BUTTER-M	COLO	3.235	PCT.	.	0.53	0/ 7	AF19	<16.037 84 >	.SHERBON.51,.811.(1968).	0.15811
423	PROTEIN	BUTTER-M	COLO	3.358	PCT.	.	1.22	0/ 7	AF12	<16.037 84 >	.SHERBON.51,.811.(1968).	0.35617
424	PROTEIN	BUTTER-M	COLO	3.735	PCT.	.	1.23	0/ 7	AF11	<16.037 84 >	.SHERBON.51,.811.(1968).	0.37496
425	PROTEIN	CHEESE-C	VOLU	3.533	PCT.	2.05	2.49	0/10	BA06A	<2.061 84 >.	.ANALYST.112,.317.(1987).	0.752737
426	PROTEIN	CHEESE-C	VOLU	3.667	PCT.	1.05	1.51	0/10	BA02A	<2.061 84 >.	.ANALYST.112,.317.(1987).	0.459053
427	PROTEIN	CHEESE-C	VOLU	3.950	PCT.	0.98	1.16	1/10	BA01A	<2.061 84 >.	.ANALYST.112,.317.(1987).	0.356611
428	PROTEIN	CHEESE-L	VOLU	2.564	PCT.	2.70	2.82	0/10	BA04A	<2.061 84 >.	.ANALYST.112,.317.(1987).	0.812339
429	PROTEIN	CHEESE-M	VOLU	3.597	PCT.	0.94	1.24	1/10	BA05A	<2.061 84 >.	.ANALYST.112,.317.(1987).	0.375875
430	PROTEIN	CHEESE-S	VOLU	3.691	PCT.	1.30	1.76	1/10	BA03A	<2.061 84 >.	.ANALYST.112,.317.(1987).	0.535573
431	PROTEIN	CREAM	COLO	2.889	PCT.	.	0.53	0/ 7	AF26	<16.037 84 >	.SHERBON.51,.811.(1968).	0.155439
432	PROTEIN	CREAM	COLO	2.933	PCT.	.	0.36	0/ 7	AF28	<16.037 84 >	.SHERBON.51,.811.(1968).	0.105825
433	PROTEIN	CREAM	COLO	2.948	PCT.	.	0.42	0/ 7	AF25	<16.037 84 >	.SHERBON.51,.811.(1968).	0.123553
434	PROTEIN	CREAM	COLO	3.028	PCT.	.	0.54	0/ 7	AF21	<16.037 84 >	.SHERBON.51,.811.(1968).	0.159496
435	PROTEIN	CREAM	COLO	3.118	PCT.	.	0.48	0/ 7	AF30	<16.037 84 >	.SHERBON.51,.811.(1968).	0.142403
436	PROTEIN	CREAM	COLO	3.168	PCT.	.	0.35	0/ 7	AF23	<16.037 84 >	.SHERBON.51,.811.(1968).	0.104085
437	PROTEIN	CREAM	COLO	3.208	PCT.	.	0.49	0/ 7	AF29	<16.037 84 >	.SHERBON.51,.811.(1968).	0.145994
438	PROTEIN	CREAM	COLO	3.210	PCT.	.	0.42	0/ 7	AF22	<16.037 84 >	.SHERBON.51,.811.(1968).	0.125149
439	PROTEIN	CREAM	COLO	3.417	PCT.	.	0.49	0/ 7	AF27	<16.037 84 >	.SHERBON.51,.811.(1968).	0.147387
440	PROTEIN	CREAM	COLO	3.752	PCT.	.	0.46	0/ 7	AF24	<16.037 84 >	.SHERBON.51,.811.(1968).	0.140326
441	PROTEIN	HALF-HAL	COLO	2.645	PCT.	.	1.17	0/ 5	AJ10A	<16.037 84 >	.SHERBON.58,.773.(1975).	0.338625
442	PROTEIN	HALF-HAL	COLO	2.663	PCT.	.	1.55	0/ 5	AJ10B	<16.037 84 >	.SHERBON.58,.773.(1975).	0.449058
443	PROTEIN	ICECREAM	COLO	3.423	PCT.	.	0.98	0/ 7	AK17	<16.037 84 >	.SHERBON.52,.138.(1969).	0.294849
444	PROTEIN	ICECREAM	COLO	3.744	PCT.	.	1.02	0/ 7	AK18	<16.037 84 >	.SHERBON.52,.138.(1969).	0.311054
445	PROTEIN	ICECREAM	COLO	3.806	PCT.	.	0.82	0/ 7	AK20	<16.037 84 >	.SHERBON.52,.138.(1969).	0.250683
446	PROTEIN	ICECREAM	COLO	3.955	PCT.	.	0.75	0/ 7	AK19	<16.037 84 >	.SHERBON.52,.138.(1969).	0.230609
447	PROTEIN	ICECREAM	COLO	4.458	PCT.	.	0.89	0/ 7	AK16	<16.037 84 >	.SHERBON.52,.138.(1969).	0.278635
448	PROTEIN	MILK	COLO	3.004	PCT.	.	0.98	0/ 5	AJ03A	<16.042 84 >	.SHERBON.58,.773.(1975).	0.289110
449	PROTEIN	MILK	COLO	3.034	PCT.	.	1.60	0/ 5	AJ03B	<16.042 84 >	.SHERBON.58,.773.(1975).	0.472735
450	PROTEIN	MILK	COLO	3.051	PCT.	.	0.88	0/ 5	AJ04A	<16.042 84 >	.SHERBON.58,.773.(1975).	0.260215
451	PROTEIN	MILK	COLO	3.065	PCT.	.	1.45	0/ 5	AJ04B	<16.042 84 >	.SHERBON.58,.773.(1975).	0.429071
452	PROTEIN	MILK	COLO	3.110	PCT.	.	1.03	0/ 5	AJ01A	<16.042 84 >	.SHERBON.58,.773.(1975).	0.305447
453	PROTEIN	MILK	COLO	3.119	PCT.	.	1.11	0/ 5	AJ07A	<16.042 84 >	.SHERBON.58,.773.(1975).	0.329320
454	PROTEIN	MILK	COLO	3.120	PCT.	.	1.30	0/ 5	AJ01B	<16.042 84 >	.SHERBON.58,.773.(1975).	0.385705
455	PROTEIN	MILK	COLO	3.136	PCT.	.	2.60	0/ 6	AI01	<16.042 84 >	.SHERBON.58,.770.(1975).	0.772021
456	PROTEIN	MILK	COLO	3.142	PCT.	.	1.48	0/ 5	AJ07B	<16.042 84 >	.SHERBON.58,.773.(1975).	0.439573
457	PROTEIN	MILK	COLO	3.144	PCT.	0.20	0.70	0/ 7	A05A	<16.037 84 >	.LUKE.50,.560.(1967).	0.207925
458	PROTEIN	MILK	COLO	3.144	PCT.	.	2.57	0/ 6	AI05	<16.042 84 >	.SHERBON.58,.770.(1975).	0.763411
459	PROTEIN	MILK	COLO	3.176	PCT.	0.42	0.74	0/ 7	A01A	<16.037 84 >	.LUKE.50,.560.(1967).	0.220151
460	PROTEIN	MILK	COLO	3.204	PCT.	.	2.32	0/ 6	AI04	<16.042 84 >	.SHERBON.58,.770.(1975).	0.691091
461	PROTEIN	MILK	COLO	3.224	PCT.	0.46	0.98	0/ 7	A09A	<16.037 84 >	.LUKE.50,.560.(1967).	0.292204
462	PROTEIN	MILK	COLO	3.236	PCT.	.	2.29	0/ 6	AI06	<16.042 84 >	.SHERBON.58,.770.(1975).	0.683187
463	PROTEIN	MILK	COLO	3.239	PCT.	.	0.62	0/ 5	AJ02A	<16.042 84 >	.SHERBON.58,.773.(1975).	0.184990
464	PROTEIN	MILK	COLO	3.243	PCT.	.	3.00	0/ 6	AI02	<16.042 84 >	.SHERBON.58,.770.(1975).	0.895282
465	PROTEIN	MILK	COLO	3.263	PCT.	.	1.45	0/ 5	AJ02B	<16.042 84 >	.SHERBON.58,.773.(1975).	0.433120
466	PROTEIN	MILK	COLO	3.372	PCT.	0.23	0.79	0/ 7	A06A	<16.037 84 >	.LUKE.50,.560.(1967).	0.237147
467	PROTEIN	MILK	COLO	3.399	PCT.	0.28	1.04	0/ 7	A08A	<16.037 84 >	.LUKE.50,.560.(1967).	0.312571
468	PROTEIN	MILK	COLO	3.424	PCT.	.	2.62	0/ 6	AI03	<16.042 84 >	.SHERBON.58,.770.(1975).	0.788321
469	PROTEIN	MILK	COLO	3.476	PCT.	.	1.86	0/ 5	AJ06A	<16.042 84 >	.SHERBON.58,.773.(1975).	0.560920
470	PROTEIN	MILK	COLO	3.507	PCT.	0.24	1.00	0/ 7	A02A	<16.037 84 >	.LUKE.50,.560.(1967).	0.301965
471	PROTEIN	MILK	COLO	3.510	PCT.	.	1.43	0/ 5	AJ06B	<16.042 84 >	.SHERBON.58,.773.(1975).	0.431875
472	PROTEIN	MILK	COLO	3.602	PCT.	.	0.80	0/ 5	AJ05A	<16.042 84 >	.SHERBON.58,.773.(1975).	0.242545
473	PROTEIN	MILK	COLO	3.610	PCT.	0.30	0.88	0/ 7	A07A	<16.037 84 >	.LUKE.50,.560.(1967).	0.266896
474	PROTEIN	MILK	COLO	3.618	PCT.	.	1.46	0/ 5	AJ05B	<16.042 84 >	.SHERBON.58,.773.(1975).	0.442952
475	PROTEIN	MILK	COLO	3.636	PCT.	0.38	0.62	0/ 7	A03A	<16.037 84 >	.LUKE.50,.560.(1967).	0.188244
476	PROTEIN	MILK	COLO	3.724	PCT.	0.31	1.22	1/ 7	A010A	<16.037 84 >	.LUKE.50,.560.(1967).	0.371741
477	PROTEIN	MILK	COLO	4.021	PCT.	0.18	0.49	1/ 7	A04A	<16.037 84 >	.LUKE.50,.560.(1967).	0.151041
478	PROTEIN	MILK	INFR	2.749	PCT.	0.45	0.87	1/ 6	AC10PR	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.253257
479	PROTEIN	MILK	INFR	2.782	PCT.	0.28	1.29	1/ 6	AC10PN	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.376194
480	PROTEIN	MILK	INFR	2.851	PCT.	0.27	0.83	0/ 6	AC09PR	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.242940
481	PROTEIN	MILK	INFR	2.872	PCT.	0.47	1.19	0/ 6	AC13PR	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.348693
482	PROTEIN	MILK	INFR	2.881	PCT.	0.98	1.29	0/ 6	AC09PN	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.378176
483	PROTEIN	MILK	INFR	2.890	PCT.	0.32	0.83	0/ 6	AC13PN	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.243439
484	PROTEIN	MILK	INFR	2.902	PCT.	0.67	1.72	0/ 6	AC01PC	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.504782
485	PROTEIN	MILK	INFR	2.902	PCT.	0.39	1.12	0/ 6	AC04PC	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.328709
486	PROTEIN	MILK	INFR	2.917	PCT.	0.40	0.88	0/ 6	AC04PB	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.258461
487	PROTEIN	MILK	INFR	2.917	PCT.	0.37	1.00	0/ 6	AC04PQ	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.293706
488	PROTEIN	MILK	INFR	2.926	PCT.	0.69	1.14	0/ 6	AC01PB	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.334983
489	PROTEIN	MILK	INFR	2.927	PCT.	0.33	1.00	0/ 6	AC04PP	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.293870
490	PROTEIN	MILK	INFR	2.928	PCT.	0.20	1.09	0/ 6	AC02PC	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.320332
491	PROTEIN	MILK	INFR	2.933	PCT.	0.81	1.16	0/ 6	AC01PQ	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.340991
492	PROTEIN	MILK	INFR	2.935	PCT.	0.65	1.40	0/ 6	AC11PR	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.411576
493	PROTEIN	MILK	INFR	2.936	PCT.	0.49	1.46	0/ 6	AC01PP	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.429233
494	PROTEIN	MILK	INFR	2.936	PCT.	0.43	0.91	0/ 6	AC11PN	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.267536
495	PROTEIN	MILK	INFR	2.940	PCT.	0.64	1.13	0/ 6	AC04PM	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.332286
496	PROTEIN	MILK	INFR	2.942	PCT.	0.50	1.16	0/ 6	AC02PQ	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.341137
497	PROTEIN	MILK	INFR	2.942	PCT.	0.37	0.92	0/ 6	AC04PL	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.270557
498	PROTEIN	MILK	INFR	2.944	PCT.	0.38	0.66	0/ 6	AC02PB	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.194120
499	PROTEIN	MILK	INFR	2.947	PCT.	0.75	1.19	0/ 6	AC01PM	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.350063
500	PROTEIN	MILK	INFR	2.957	PCT.	0.34	1.00	0/ 6	AC02PM	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.294309
501	PROTEIN	MILK	INFR	2.958	PCT.	0.28	0.79	0/ 6	AC15PR	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.232523
502	PROTEIN	MILK	INFR	2.959	PCT.	0.41	0.41	1/ 6	AC15PN	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.120681
503	PROTEIN	MILK	INFR	2.960	PCT.	0.51	0.84	0/ 6	AC01PL	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.247258
504	PROTEIN	MILK	INFR	2.970	PCT.	0.55	1.02	0/ 6	AC02PL	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.300398
505	PROTEIN	MILK	INFR	2.982	PCT.	0.43	2.18	0/ 6	AC02PP	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.642406
506	PROTEIN	MILK	INFR	3.037	PCT.	0.33	0.93	0/ 6	AC05PC	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.274809
507	PROTEIN	MILK	INFR	3.040	PCT.	0.45	0.98	0/ 6	AC03PC	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.289632
508	PROTEIN	MILK	INFR	3.041	PCT.	0.10	1.02	1/ 6	AC16PR	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.301468
509	PROTEIN	MILK	INFR	3.051	PCT.	0.21	0.88	0/ 6	AC03PQ	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.260217
510	PROTEIN	MILK	INFR	3.054	PCT.	0.25	1.07	0/ 6	AC05PQ	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.316452
511	PROTEIN	MILK	INFR	3.057	PCT.	0.31	0.85	0/ 6	AC03PB	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.251428
512	PROTEIN	MILK	INFR	3.058	PCT.	0.44	1.13	0/ 6	AC03PP	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.334265
513	PROTEIN	MILK	INFR	3.061	PCT.	0.43	1.07	0/ 6	AC05PP	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.316556
514	PROTEIN	MILK	INFR	3.063	PCT.	0.31	0.93	0/ 6	AC05PB	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.275160
515	PROTEIN	MILK	INFR	3.063	PCT.	0.25	0.73	0/ 6	AC05PM	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.215986
516	PROTEIN	MILK	INFR	3.063	PCT.	0.31	1.55	0/ 6	AC26PS	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.458599
517	PROTEIN	MILK	INFR	3.066	PCT.	0.34	0.73	0/ 6	AC16PN	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.216021
518	PROTEIN	MILK	INFR	3.070	PCT.	.	2.98	0/ 5	AM03D	<16.083 84 >	.BIGGS.55,.488.(1972).	0.882019
519	PROTEIN	MILK	INFR	3.077	PCT.	0.41	0.97	0/ 6	AC05PL	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.287206
520	PROTEIN	MILK	INFR	3.079	PCT.	0.36	0.99	0/ 6	AC03PM	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.293151
521	PROTEIN	MILK	INFR	3.087	PCT.	0.41	1.06	0/ 6	AC03PL	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.314007
522	PROTEIN	MILK	INFR	3.090	PCT.	.	2.77	0/ 5	AM04D	<16.083 84 >	.BIGGS.55,.488.(1972).	0.820665
523	PROTEIN	MILK	INFR	3.091	PCT.	0.69	1.37	0/ 6	AC26PQ	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.405905
524	PROTEIN	MILK	INFR	3.092	PCT.	0.38	1.59	0/ 6	AC20PS	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.471125
525	PROTEIN	MILK	INFR	3.099	PCT.	0.31	1.57	0/ 6	AC20PQ	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.465350

OBS	ANALYTE	MATRIX	METHOD	CONCN	UNITS	RSDO	RSDR	DROPPED/ # LABS	CODE	METHOD NO.	REFERENCE	HORRAT
527	PROTEIN	MILK	INFR	3.102	PCT.	.	2.34	0/ 4	AM08D	<16.083 84 >	.BIGGS.55,.488.(1972).	0.693691
528	PROTEIN	MILK	INFR	3.105	PCT.	0.26	1.22	0/ 6	AC23PS	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.361712
529	PROTEIN	MILK	INFR	3.117	PCT.	0.24	1.82	0/ 6	AC19PS	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.539929
530	PROTEIN	MILK	INFR	3.123	PCT.	0.39	1.38	0/ 6	AC14PR	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.409512
531	PROTEIN	MILK	INFR	3.124	PCT.	0.48	1.34	0/ 6	AC19PO	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.397658
532	PROTEIN	MILK	INFR	3.125	PCT.	0.18	1.53	0/ 6	AC23PO	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.454061
533	PROTEIN	MILK	INFR	3.135	PCT.	0.52	1.67	0/ 5	AC25PS	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.495847
534	PROTEIN	MILK	INFR	3.140	PCT.	0.84	2.34	1/ 6	AC27PS	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.694946
535	PROTEIN	MILK	INFR	3.141	PCT.	0.33	0.93	0/ 6	AC14PN	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.276208
536	PROTEIN	MILK	INFR	3.149	PCT.	0.33	1.79	1/ 6	AC28PS	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.531833
537	PROTEIN	MILK	INFR	3.152	PCT.	0.47	1.58	0/ 6	AC25PO	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.469499
538	PROTEIN	MILK	INFR	3.152	PCT.	0.49	2.46	1/ 6	AC18PS	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.731004
539	PROTEIN	MILK	INFR	3.155	PCT.	0.37	1.76	0/ 6	AC22PS	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.523070
540	PROTEIN	MILK	INFR	3.158	PCT.	0.22	1.56	0/ 6	AC28PO	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.463704
541	PROTEIN	MILK	INFR	3.162	PCT.	0.40	2.71	0/ 6	AC24PS	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.805696
542	PROTEIN	MILK	INFR	3.165	PCT.	0.18	1.63	0/ 6	AC22PO	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.484665
543	PROTEIN	MILK	INFR	3.177	PCT.	0.45	2.47	0/ 6	AC24PO	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.734837
544	PROTEIN	MILK	INFR	3.202	PCT.	0.39	2.61	0/ 6	AC18PO	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.777435
545	PROTEIN	MILK	INFR	3.206	PCT.	0.30	2.28	0/ 6	AC27PO	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.679245
546	PROTEIN	MILK	INFR	3.210	PCT.	0.49	1.06	0/ 6	AC06PC	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.315851
547	PROTEIN	MILK	INFR	3.220	PCT.	0.61	2.14	0/ 6	AC21PO	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.637960
548	PROTEIN	MILK	INFR	3.220	PCT.	.	1.77	0/ 5	AM01D	<16.083 84 >	.BIGGS.55,.488.(1972).	0.527659
549	PROTEIN	MILK	INFR	3.224	PCT.	0.35	1.40	0/ 6	AC06PQ	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.417439
550	PROTEIN	MILK	INFR	3.233	PCT.	0.33	1.20	0/ 6	AC06PP	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.357957
551	PROTEIN	MILK	INFR	3.236	PCT.	0.39	0.94	0/ 6	AC06PB	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.280433
552	PROTEIN	MILK	INFR	3.236	PCT.	0.20	0.85	0/ 6	AC06PM	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.253583
553	PROTEIN	MILK	INFR	3.237	PCT.	0.43	2.68	0/ 5	AC21PS	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.799574
554	PROTEIN	MILK	INFR	3.237	PCT.	.	2.05	0/ 4	AM06D	<16.083 84 >	.BIGGS.55,.488.(1972).	0.611629
555	PROTEIN	MILK	INFR	3.255	PCT.	0.60	1.51	0/ 6	AC12PR	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.450883
556	PROTEIN	MILK	INFR	3.262	PCT.	0.48	1.10	0/ 6	AC12PN	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.328559
557	PROTEIN	MILK	INFR	3.268	PCT.	0.40	1.37	0/ 6	AC06PL	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.409331
558	PROTEIN	MILK	INFR	3.413	PCT.	0.34	1.31	0/ 6	AC17PR	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.393970
559	PROTEIN	MILK	INFR	3.429	PCT.	0.30	1.12	0/ 6	AC17PN	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.337064
560	PROTEIN	MILK	INFR	3.454	PCT.	.	1.91	0/ 5	AM05D	<16.083 84 >	.BIGGS.55,.488.(1972).	0.575439
561	PROTEIN	MILK	INFR	3.522	PCT.	0.49	1.59	0/ 6	AC07PQ	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.480431
562	PROTEIN	MILK	INFR	3.522	PCT.	0.32	1.00	0/ 6	AC07PC	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.302169
563	PROTEIN	MILK	INFR	3.540	PCT.	.	1.11	0/ 4	AM10D	<16.083 84 >	.BIGGS.55,.488.(1972).	0.335657
564	PROTEIN	MILK	INFR	3.541	PCT.	0.86	2.13	0/ 6	AC07PP	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.644122
565	PROTEIN	MILK	INFR	3.542	PCT.	0.51	1.56	0/ 6	AC08PQ	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.471785
566	PROTEIN	MILK	INFR	3.549	PCT.	0.27	0.95	0/ 6	AC07PB	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.287386
567	PROTEIN	MILK	INFR	3.551	PCT.	0.47	0.86	0/ 6	AC08PC	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.260179
568	PROTEIN	MILK	INFR	3.554	PCT.	0.35	1.39	0/ 6	AC07PM	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.420580
569	PROTEIN	MILK	INFR	3.566	PCT.	0.39	1.12	0/ 6	AC07PL	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.339052
570	PROTEIN	MILK	INFR	3.568	PCT.	0.32	1.44	0/ 6	AC08PM	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.435970
571	PROTEIN	MILK	INFR	3.579	PCT.	0.21	1.35	0/ 6	AC08PP	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.408909
572	PROTEIN	MILK	INFR	3.587	PCT.	0.74	1.26	0/ 6	AC08PL	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.381782
573	PROTEIN	MILK	INFR	3.592	PCT.	0.31	1.09	0/ 6	AC08PB	<16.083 84 >	.BIGGS.61,.1015.(1978).	0.330341
574	PROTEIN	MILK	INFR	3.794	PCT.	.	2.08	0/ 5	AM02D	<16.083 84 >	.BIGGS.55,.488.(1972).	0.635574
575	PROTEIN	MILK	INFR	3.850	PCT.	.	0.95	0/ 4	AM07D	<16.083 84 >	.BIGGS.55,.488.(1972).	0.290927
576	PROTEIN	MILK	VOLU	2.252	PCT.	0.46	1.07	0/17	BJ06A	<16.036 84 >	.GRAPPIN.71,0893(1988).	0.302270
577	PROTEIN	MILK	VOLU	2.495	PCT.	0.60	0.84	0/17	BJ05A	<16.036 84 >	.GRAPPIN.71,0893(1988).	0.240976
578	PROTEIN	MILK	VOLU	3.062	PCT.	0.66	1.09	0/20	BJ04A	<16.036 84 >	.GRAPPIN.71,0893(1988).	0.322497
579	PROTEIN	MILK	VOLU	3.095	PCT.	.	2.56	0/ 4	AM08C	<2.055 84 >.	.BIGGS.55,.488.(1972).	0.758633
580	PROTEIN	MILK	VOLU	3.100	PCT.	.	1.24	0/ 4	AM09C	<2.055 84 >.	.BIGGS.55,.488.(1972).	0.367552
581	PROTEIN	MILK	VOLU	3.101	PCT.	0.61	1.19	0/20	BJ03A	<16.036 84 >	.GRAPPIN.71,0893(1988).	0.352743
582	PROTEIN	MILK	VOLU	3.217	PCT.	.	1.65	0/ 4	AM06C	<2.055 84 >.	.BIGGS.55,.488.(1972).	0.491828
583	PROTEIN	MILK	VOLU	3.470	PCT.	.	2.01	0/ 4	AM10C	<2.055 84 >.	.BIGGS.55,.488.(1972).	0.605988
584	PROTEIN	MILK	VOLU	3.777	PCT.	.	1.81	0/ 4	AM07C	<2.055 84 >.	.BIGGS.55,.488.(1972).	0.55271
585	PROTEIN	MILK	VOLU	4.102	PCT.	1.01	1.04	0/18	BJ02A	<16.036 84 >	.GRAPPIN.71,0893(1988).	0.32155
586	PROTEIN	MILK	VOLU	4.338	PCT.	0.30	0.94	0/18	BJ01A	<16.036 84 >	.GRAPPIN.71,0893(1988).	0.29309
587	PROTEIN	MILK-CHO	COLO	2.905	PCT.	.	0.95	0/ 5	AJ09A	<16.037 84 >	.SHERBON.58,.773.(1975).	0.27886
588	PROTEIN	MILK-CHO	COLO	2.935	PCT.	.	1.36	0/ 5	AJ09B	<16.037 84 >	.SHERBON.58,.773.(1975).	0.39982
589	PROTEIN	MILK-CHO	COLO	2.957	PCT.	.	0.30	0/ 7	AF9	<16.037 84 >	.SHERBON.51,.811.(1968).	0.08829
590	PROTEIN	MILK-CHO	COLO	3.015	PCT.	.	0.73	0/ 7	AF4	<16.037 84 >	.SHERBON.51,.811.(1968).	0.21547
591	PROTEIN	MILK-CHO	COLO	3.076	PCT.	.	1.69	0/ 6	AI07	<16.037 84 >	.SHERBON.58,.770.(1975).	0.50034
592	PROTEIN	MILK-CHO	COLO	3.126	PCT.	.	0.25	0/ 7	AF8	<16.037 84 >	.SHERBON.51,.811.(1968).	0.07420
593	PROTEIN	MILK-CHO	COLO	3.131	PCT.	.	0.57	0/ 7	AF5	<16.037 84 >	.SHERBON.51,.811.(1968).	0.16921
594	PROTEIN	MILK-CHO	COLO	3.182	PCT.	.	0.36	0/ 7	AF1	<16.037 84 >	.SHERBON.51,.811.(1968).	0.10713
595	PROTEIN	MILK-CHO	COLO	3.185	PCT.	.	0.44	0/ 7	AF3	<16.037 84 >	.SHERBON.51,.811.(1968).	0.13095
596	PROTEIN	MILK-CHO	COLO	3.225	PCT.	.	0.60	0/ 7	AF7	<16.037 84 >	.SHERBON.51,.811.(1968).	0.17891
597	PROTEIN	MILK-CHO	COLO	3.466	PCT.	.	0.26	0/ 7	AF10	<16.037 84 >	.SHERBON.51,.811.(1968).	0.07837
598	PROTEIN	MILK-CHO	COLO	3.670	PCT.	.	0.35	0/ 7	AF6	<16.037 84 >	.SHERBON.51,.811.(1968).	0.10641
599	PROTEIN	MILK-CHO	COLO	3.702	PCT.	.	0.53	0/ 7	AF2	<16.037 84 >	.SHERBON.51,.811.(1968).	0.16135
600	PROTEIN	NFDMS	COLO	34.445	PCT.	.	0.80	0/ 7	AK08	<16.037 84 >	.SHERBON.52,.138.(1969).	0.34071
601	PROTEIN	NFDMS	COLO	34.449	PCT.	.	0.44	0/ 7	AK15	<16.037 84 >	.SHERBON.52,.138.(1969).	0.18740
602	PROTEIN	NFDMS	COLO	34.640	PCT.	.	2.12	0/ 7	AK04	<16.037 84 >	.SHERBON.52,.138.(1969).	0.90366
603	PROTEIN	NFDMS	COLO	34.802	PCT.	.	0.75	0/ 7	AK11	<16.037 84 >	.SHERBON.52,.138.(1969).	0.31992
604	PROTEIN	NFDMS	COLO	34.841	PCT.	.	0.51	0/ 7	AK12	<16.037 84 >	.SHERBON.52,.138.(1969).	0.21758
605	PROTEIN	NFDMS	COLO	34.957	PCT.	.	0.49	0/ 7	AK03	<16.037 84 >	.SHERBON.52,.138.(1969).	0.20915
606	PROTEIN	NFDMS	COLO	35.061	PCT.	.	0.67	0/ 7	AK07	<16.037 84 >	.SHERBON.52,.138.(1969).	0.28611
607	PROTEIN	NFDMS	COLO	35.363	PCT.	.	0.64	0/ 7	AK06	<16.037 84 >	.SHERBON.52,.138.(1969).	0.27365
608	PROTEIN	NFDMS	COLO	35.403	PCT.	.	2.11	0/ 7	AK02	<16.037 84 >	.SHERBON.52,.138.(1969).	0.90235
609	PROTEIN	NFDMS	COLO	35.424	PCT.	.	0.88	0/ 7	AK09	<16.037 84 >	.SHERBON.52,.138.(1969).	0.37637
610	PROTEIN	NFDMS	COLO	35.496	PCT.	.	1.32	0/ 7	AK10	<16.037 84 >	.SHERBON.52,.138.(1969).	0.56473
611	PROTEIN	NFDMS	COLO	35.640	PCT.	.	0.61	0/ 7	AK13	<16.037 84 >	.SHERBON.52,.138.(1969).	0.26113
612	PROTEIN	NFDMS	COLO	35.754	PCT.	.	0.52	0/ 7	AK14	<16.037 84 >	.SHERBON.52,.138.(1969).	0.22271
613	PROTEIN	NFDMS	COLO	36.527	PCT.	.	0.89	0/ 7	AK05	<16.037 84 >	.SHERBON.52,.138.(1969).	0.38241
614	SALT	BUTTER	VOLU	0.997	PCT.	2.46	3.36	0/ 7	AW2B	<16.235 84 >	.WEIK.49,.518.(1966).	0.83964
615	SALT	BUTTER	VOLU	1.291	PCT.	1.01	2.77	0/ 7	AW5B	<16.235 84 >	.WEIK.49,.518.(1966).	0.71968
616	SALT	BUTTER	VOLU	1.509	PCT.	0.71	2.12	0/ 7	AW7B	<16.235 84 >	.WEIK.49,.518.(1966).	0.56384
617	SALT	BUTTER	VOLU	2.045	PCT.	0.29	1.39	0/ 7	AW1B	<16.235 84 >	.WEIK.49,.518.(1966).	0.38701
618	SALT	BUTTER	VOLU	2.245	PCT.	0.41	2.38	1/ 7	AW3B	<16.235 84 >	.WEIK.49,.518.(1966).	0.67202
619	SALT	BUTTER	VOLU	2.399	PCT.	0.40	1.78	1/ 7	AW4B	<16.235 84 >	.WEIK.49,.518.(1966).	0.50765
620	SALT+CURD	BUTTER	GRAV	2.327	PCT.	1.84	2.75	0/ 6	BD6B	<16.232 84 >	.WEBER.35,.194.(1952).	0.78068
621	SALT+CURD	BUTTER	GRAV	2.359	PCT.	1.45	2.79	0/ 6	B04B	<16.232 84 >	.WEBER.35,.194.(1952).	0.79368
622	SALT+CURD	BUTTER	GRAV	2.363	PCT.	1.39	1.77	0/ 6	BD2B	<16.232 84 >	.WEBER.35,.194.(1952).	0.50366
623	SALT+CURD	BUTTER	GRAV	2.368	PCT.	1.48	2.69	0/ 6	BD5B	<16.232 84 >	.WEBER.35,.194.(1952).	0.76566
624	SALT+CURD	BUTTER	GRAV	2.371	PCT.	1.58	2.94	0/ 6	BD1B	<16.232 84 >	.WEBER.35,.194.(1952).	0.83698
625	SALT+CURD	BUTTER	GRAV	2.439	PCT.	1.08	2.12	1/ 6	BD3B	<16.232 84 >	.WEBER.35,.194.(1952).	0.60613
626	SODIUM	MILK-DRI	ATAB	0.302	PCT.	2.68	7.51	0/ 7	AT01A	<IDF119:1984	6, 1985 IDFBUL207 6,133(1986)	1.56816
627	SODIUM	MILK-DRI	ATAB	0.341	PCT.	1.48	7.49	0/ 7	AT02A	<IDF119:1984	6, 1985 IDFBUL207 6,133(1986)	1.59250
628	SODIUM	MILK-DRI	ATAB	0.439	PCT.	1.81	1.84	1/ 7	AT03A	<IDF119:1984	6, 1985 IDFBUL207 6,133(1986)	0.40639
629	SODIUM	MILK-DRI	ATAB	0.442	PCT.	2.69	2.79	1/ 7	AT04A	<IDF119:1984	6, 1985 IDFBUL207 6,133(1986)	0.61677
630	SODIUM	MILK-DRI	ATAB	0.477	PCT.	1.64	2.25	1/ 7	AT05A	<IDF119:1984	6, 1985 IDFBUL207 6,133(1986)	0.50326
631	SODIUM	MILK-DRI	ATAB	0.480	PCT.	2.40	2.58	1/ 7	AT06A	<IDF119:1984	6, 1985 IDFBUL207 6,133(1986)	0.57758
632	SOLIDS	CREAM	GRAV	22.872	PCT.	0.12	0.25	0/12	AQ7A	<IDF21A:1982	.IDFBUL207.2,10(1986).	0.10011
633	SOLIDS	CREAM	GRAV	23.060	PCT.	0.26	0.30	0/12	AQ8A	<IDF21A:1982	.IDFBUL207.2,10(1986).	0.12028
634	SOLIDS	CREAM	GRAV	31.919	PCT.	0.61	0.99	0/12	AQ9A	<IDF21A:1982	.IDFBUL207.2,10(1986).	0.41683
635	SOLIDS	CREAM	GRAV	32.368	PCT.	0.15	0.32	1/12	AQ10A	<IDF21A:1982	.IDFBUL207.2,10(1986).	0.13502
636	SOLIDS	CREAM	GRAV	41.002	PCT.	0.14	0.24	0/12	AQ11A	<IDF21A:1982	.IDFBUL207.2,10(1986).	0.10493
637	SOLIDS	CREAM	GRAV	41.499	PCT.	0.19	0.28	0/12	AQ12A	<IDF21A:1982	.IDFBUL207.2,10(1986).	0.122641
638	SOLIDS	MILK	GRAV	9.094	PCT.	0.33	0.73	1/13	AQ1A	<IDF21A:1982	.IDFBUL207.2,10(1986).	0.254432
639	SOLIDS	MILK	GRAV	9.197	PCT.	0.64	0.83	0/13	AQ2A	<IDF21A:1982	.IDFBUL207.2,10(1986).	0.289775
640	SOLIDS	MILK	GRAV	11.402	PCT.	0.29	0.60	0/13	AQ3A	<IDF21A:1982	.IDFBUL207.2,10(1986).	0.216362
641	SOLIDS	MILK	GRAV	11.685	PCT.	0.27	0.45	0/13	AQ4A	<IDF21A:1982	.IDFBUL207.2,10(1986).	0.162873
642	SOLIDS	MILK	GRAV	12.532	PCT.	0.46	0.61	0/13	AQ5A	<IDF21A:1982	.IDFBUL207.2,10(1986).	0.223121
643	SOLIDS	MILK	GRAV	12.588	PCT.	0.20	0.58	1/13	AQ6A	<IDF21A:1982	.IDFBUL207.2,10(1986).	0.212289
644	SOLIDS	MILK-EVA	GRAV	25.091	PCT.	0.45	0.62	0/13	AQ13A	<IDF21A:1982	.IDFBUL207.2,10(1986).	0.251756

OBS	ANALYTE	MATRIX	METHOD	CONCN	UNITS	RSDO	RSDR	DROPPED/ # LABS	CODE	METHOD NO.	REFERENCE	HORRAT
645	SOLIDS	MILK-EVA	GRAV	25.097	PCT.	0.32	0.62	0/13	AQ14A	<IDF21A:1982	.IDFBUL207.2,10(1986).	0.251765
646	SOLIDS	MILK-EVA	GRAV	25.104	PCT.	0.57	0.68	1/13	AQ15A	<IDF21A:1982	.IDFBUL207.2,10(1986).	0.276141
647	SOLIDS	MILK-EVA	GRAV	25.171	PCT.	0.28	0.60	0/13	AQ16A	<IDF21A:1982	.IDFBUL207.2,10(1986).	0.243751
648	SOLIDS	MILK-EVA	GRAV	25.443	PCT.	0.54	0.59	0/13	AQ17A	<IDF21A:1982	.IDFBUL207.2,10(1986).	0.240078
649	SOLIDS	MILK-EVA	GRAV	26.232	PCT.	0.12	0.53	0/ 8	AG2A3	<XXII8 35 3H	.FRARY.23,.453.(1940).	0.216657
650	SOLIDS	MILK-EVA	GRAV	26.244	PCT.	0.13	0.81	0/ 7	AG2B3	<BATH/AIR.3.	.FRARY.23,.453.(1940).	0.331140
651	SOLIDS	MILK-EVA	GRAV	26.264	PCT.	0.14	0.29	0/ 8	AG2C3	<BATH/VAC.3.	.FRARY.23,.453.(1940).	0.118570
652	SOLIDS	MILK-EVA	GRAV	26.267	PCT.	0.18	0.78	0/ 7	AG2A2	<XXII8 35 2H	.FRARY.23,.453.(1940).	0.318917
653	SOLIDS	MILK-EVA	GRAV	26.304	PCT.	0.12	0.34	0/ 8	AG2C2	<BATH/VAC.2.	.FRARY.23,.453.(1940).	0.139044
654	SOLIDS	MILK-EVA	GRAV	26.313	PCT.	0.11	0.81	0/ 7	AG2B2	<BATH/AIR.2.	.FRARY.23,.453.(1940).	0.331270
655	SOLIDS	MILK-EVA	GRAV	26.712	PCT.	0.15	1.55	0/ 7	AG1B3	<BATH/AIR.3.	.FRARY.23,.453.(1940).	0.635350
656	SOLIDS	MILK-EVA	GRAV	26.821	PCT.	0.19	0.68	1/ 8	AG1A3	<XXII8 35 3H	.FRARY.23,.453.(1940).	0.278904
657	SOLIDS	MILK-EVA	GRAV	26.845	PCT.	0.15	0.27	0/ 8	AG1C3	<BATH/VAC.3.	.FRARY.23,.453.(1940).	0.110756
658	SOLIDS	MILK-EVA	GRAV	26.875	PCT.	0.06	1.11	1/ 7	AG1B2	<BATH/AIR.2.	.FRARY.23,.453.(1940).	0.455409
659	SOLIDS	MILK-EVA	GRAV	26.882	PCT.	0.15	0.77	1/ 8	AG1A2	<XXII8 35 2H	.FRARY.23,.453.(1940).	0.315927
660	SOLIDS	MILK-EVA	GRAV	26.882	PCT.	0.11	0.27	0/ 8	AG1C2	<BATH/VAC.2.	.FRARY.23,.453.(1940).	0.110780
661	SOLIDS	MILK-EVA	GRAV	33.286	PCT.	0.42	0.78	0/13	AQ18A	<IDF21A:1982	.IDFBUL207.2,10(1986).	0.330489

editions of *Official Methods of Analysis* used a Roman numeral for the chapter followed by an Arabic consecutive number. IDF methods are preceded by "IDF" followed by the method number and year. The "REFERENCE" column presents the last name of the first author and the AOAC *Journal* reference (if source is not indicated), a reference to a different journal (i.e., *Analyst*), or the IDF reference (prefixed IDF). The final column ("HORRAT") has been found very useful for interpreting the acceptability of assays. It is the ratio of the value given in the RSD_R column to the value calculated from the Horwitz formula $RSD_R = 2 \exp(1 - 0.5 \log_{10}C)$, where C is the value in the CONCN column transformed to a decimal fraction, e.g., 1% = 0.01. A value greater than 2 usually indicates an unacceptable assay—one that is too variable for most analytical purposes.

A similar database (Database 2) was prepared of 91 assays that were not accepted for reasons indicated by letters in a "STAR" column: (*1*) "I" for impurity analysis, e.g., fat in casein; (*2*) "N" for not applicable, e.g., fat in skim milk by the infrared method; (*3*) "P" for a proficiency study (not a collaborative study); (*4*) "R" for a rejected method, usually because of poor precision; (*5*) "S" for use of an individual standard when a common standard should have been used in the infrared method for milk constituents; and (*6*) "*" for an AOAC method designated surplus. These special cases were not included in the calculation of the summary statistics for the reasons given.

Subsequent tables summarize the data by analyte and method for various products. When a number (N) of assays, often designated in the original papers as "samples," were reported in a single study, the data were recalculated assay-by-assay, and then the mean and precision parameters were averaged for products with similar analyte concentrations when the precision parameters were comparable, furnishing an average concentration, an average RSD_r, and an average RSD_R. Later tables further summarize the data by analytes and by methods in those cases where a sufficient number of assays were available to provide enough data for determining the 90% limits of the RSD_R distribution. The means (when comparable concentrations were present) and RSD_r and RSD_R values are reported with a ± standard deviation that merely reflects the dispersion of the RSD_R estimates; it is not used as a precision parameter of the reported estimate. The utility of using the simple average for compositing the RSD_R data was affirmed by the observation that this value was the same as the result of utilizing the 2-way ANOVA on the entire set of assays or the average of the 1-way ANOVA on each assay of the set. The 2-way ANOVA procedure or weighting variances or their relative values by the degrees of freedom might in some cases be more desirable. However, the analysis of multiple studies showed that estimated individual RSD_r or RSD_R values of the same chemical method for the same commodity at the same analyte level can vary by a factor of 2 or even 3. These differences in parameters probably reflect differences in the performance by collaborators involved in the separate studies. As stated above, we were investigating the violations of the assumption of a fixed, invariant precision for an analytical method under fixed circumstances except for the performance of the individual collaborators.

Some studies replicated the same test materials as blind duplicates. Ordinarily such assays are combined in order to obtain an estimate of within-laboratory precision (8). In the present study, the dispersion of the between-laboratory precision term was of greater interest, so the individual results from blind duplicates were not combined.

In calculating an overall average concentration from a series of assays, a few unrelated values may be omitted to avoid distortions. An example is the omission of the protein content (35 g/100 g) of a single nonfat dry milk in calculating the average concentration of protein in a series consisting of 13 liquid products with a content of 2–4 g protein/100 g and the 1 nonfat dry milk. There was no need to omit the RSD_R of this matrix since its inclusion or omission did not affect this parameter. Throughout this paper, absolute concentrations are expressed in terms of g analyte/100 g matrix (product) rather than in terms of percent, to avoid confusion with relative RSD_R values, which are expressed in terms of percent (%).

Several different ways of summarizing and presenting the precision parameters, RSD_r and RSD_R, from multiple collaborative assays and multiple studies were examined. Statistical functions using the standard deviation directly were not considered acceptable since such expressions would inevitably be interpreted by chemists in terms of the normal distribution. Precision estimates appear to obey no clearly discernible law except, very roughly, the Horwitz formula (10). No attempt was made to fit a mathematical model to the frequency histograms. Therefore, the empirical distributions presented here can be used only as rough clues as to what to expect in the future. However rough these predictions may be, they do show how erratic a population of precision estimates can be. About all that can be said is that the RSD_R values within a study show a tendency to be close to one another, and consequently to be distant from the cluster of estimates from other comparable studies. Therefore, a conservative approach was used to characterize the distributions of RSD_r and RSD_R.

Cumulative distribution functions (CDFs).—In situations in which a distribution is unknown, and particularly when it is asymmetrical, the distribution of estimates is best described by an empirical CDF. For the present case, this function is *the percentage RSD_R estimates less than or equal to given RSD_R values* plotted against the given *RSD_R values*. The cumulative percentages are calculated from the available RSD_R estimates arranged from lowest to highest. For

Database 2. Database of unaccepted assays for standard methods for examination of dairy products

OBS	ANALYTE	MATRIX	METHOD	CONCN	UNITS	RSDO	RSDR	DROPPED/ # LABS	CODE	METHOD NO.	REFERENCE	HORRAT	STAR	
1	CALCIUM	MILK-DRI	ATAB	0.980	PCT.	1.32	7.85	0/ 6	AT04C	<IDF119:1984	6, 1985 IDFBUL207 6,133(1986)	.		R
2	CALCIUM	MILK-DRI	ATAB	0.789	PCT.	2.96	6.13	0/ 6	AT01C	<IDF119:1984	6, 1985 IDFBUL207 6,133(1986)	1.4788		R
3	CALCIUM	MILK-DRI	ATAB	0.801	PCT.	1.72	6.00	0/ 6	AT02C	<IDF119:1984	6, 1985 IDFBUL207 6,133(1986)	1.4506		R
4	CALCIUM	MILK-DRI	ATAB	0.976	PCT.	0.79	8.13	0/ 6	AT03C	<IDF119:1984	6, 1985 IDFBUL207 6,133(1986)	2.0251		R
5	CALCIUM	MILK-DRI	ATAB	1.127	PCT.	0.76	6.72	0/ 6	AT05C	<IDF119:1984	6, 1985 IDFBUL207 6,133(1986)	1.7106		R
6	CALCIUM	MILK-DRI	ATAB	1.146	PCT.	0.74	7.38	0/ 6	AT06C	<IDF119:1984	6, 1985 IDFBUL207 6,133(1986)	1.8833		R
7	FAT	CASEIN	GRAV	0.961	PCT.	6.59	6.59	0/ 6	AU02	<16.284	84 > .EISSES.IDF235,0067(1988).	1.6376		I
8	FAT	CASEIN	GRAV	1.234	PCT.	4.49	4.49	0/ 6	AU01	<16.284	84 > .EISSES.IDF235,0067(1988).	1.1586		I
9	FAT	CASEIN	GRAV	1.339	PCT.	5.02	5.75	0/ 6	AU03	<16.284	84 > .EISSES.IDF235,0067(1988).	1.5021		I
10	FAT	CASEIN-R	GRAV	0.347	PCT.	8.05	12.16	0/ 6	AU08	<16.284	84 > .EISSES.IDF235,0067(1988).	2.5926		I
11	FAT	CASEIN-R	GRAV	0.478	PCT.	8.49	13.27	0/ 6	AU09	<16.284	84 > .EISSES.IDF235,0067(1988).	2.9690		I
12	FAT	CASEIN-R	GRAV	0.575	PCT.	6.58	7.79	0/ 6	AU07	<16.284	84 > .EISSES.IDF235,0067(1988).	1.7919		I
13	FAT	CASEINAT	GRAV	0.957	PCT.	3.19	8.74	0/ 6	AU06	<16.284	84 > .EISSES.IDF235,0067(1988).	2.1705		I
14	FAT	CASEINAT	GRAV	1.047	PCT.	3.57	4.56	0/ 6	AU05	<16.284	84 > .EISSES.IDF235,0067(1988).	1.1479		I
15	FAT	CASEINAT	GRAV	1.100	PCT.	2.79	8.83	0/ 6	AU04	<16.284	84 > .EISSES.IDF235,0067(1988).	2.2394		I
16	FAT	MILK	INFR	1.978	PCT.	0.80	2.47	0/ 6	AC28FK	<16.083	84 > .BIGGS.61,..015.(1978).	0.6843		N
17	FAT	MILK	INFR	1.980	PCT.	0.90	2.53	0/ 6	AC22FK	<16.083	84 > .BIGGS.61,..015.(1978).	0.7010		N
18	FAT	MILK	INFR	1.988	PCT.	1.01	3.23	0/ 6	AC22FG	<16.083	84 > .BIGGS.61,..015.(1978).	0.8955		N
19	FAT	MILK	INFR	1.996	PCT.	0.93	3.23	0/ 6	AC28FG	<16.083	84 > .BIGGS.61,..015.(1978).	0.8960		N
20	FAT	MILK	INFR	2.021	PCT.	1.12	5.42	0/ 6	AC19FK	<16.083	84 > .BIGGS.61,..015.(1978).	1.5064		N
21	FAT	MILK	INFR	2.035	PCT.	1.28	2.48	0/ 6	AC25FG	<16.083	84 > .BIGGS.61,..015.(1978).	0.6900		N
22	FAT	MILK	INFR	2.050	PCT.	1.77	4.24	0/ 5	AC25FK	<16.083	84 > .BIGGS.61,..015.(1978).	1.1810		N
23	FAT	MILK	INFR	2.086	PCT.	0.77	3.82	0/ 6	AC19FG	<16.083	84 > .BIGGS.61,..015.(1978).	1.0667		N
24	FAT	MILK	INFR	2.710	PCT.	0.67	6.18	0/ 6	AC01FA	<16.083	84 > .BIGGS.61,..015.(1978).	1.7951		S
25	FAT	MILK	INFR	2.782	PCT.	0.31	5.63	0/ 6	AC04FA	<16.083	84 > .BIGGS.61,..015.(1978).	1.6419		S
26	FAT	MILK	INFR	3.126	PCT.	0.16	5.13	0/ 6	AC02FA	<16.083	84 > .BIGGS.61,..015.(1978).	1.5225		S
27	FAT	MILK	INFR	3.476	PCT.	0.19	4.82	0/ 6	AC03FA	<16.083	84 > .BIGGS.61,..015.(1978).	1.4535		S
28	FAT	MILK	INFR	3.499	PCT.	0.18	4.80	0/ 6	AC05FA	<16.083	84 > .BIGGS.61,..015.(1978).	1.4490		S
29	FAT	MILK	INFR	3.961	PCT.	0.22	4.35	0/ 6	AC06FA	<16.083	84 > .BIGGS.61,..015.(1978).	1.3378		S
30	FAT	MILK	INFR	4.759	PCT.	0.32	4.09	0/ 6	AC07FA	<16.083	84 > .BIGGS.61,..015.(1978).	1.2931		S
31	FAT	MILK	INFR	4.934	PCT.	0.23	3.64	0/ 6	AC08FA	<16.083	84 > .BIGGS.61,..015.(1978).	1.1571		S
32	FAT	MILK	VOLU	1.842	PCT.	1.22	4.63	0/ 6	AN06C	<16.055	70 > .MITCHELL.50,..537.(1967).	1.2690		*
33	FAT	MILK	VOLU	1.852	PCT.	1.15	4.61	0/ 6	AN08C	<16.055	70 > .MITCHELL.50,..537.(1967).	1.2645		*
34	FAT	MILK	VOLU	1.950	PCT.	3.47	5.14	0/ 6	AN08B	<16.065	84 > .MITCHELL.50,..537.(1967).	1.4209		N
35	FAT	MILK	VOLU	1.995	PCT.	0.79	3.41	1/ 6	AN06B	<16.065	84 > .MITCHELL.50,..537.(1967).	0.9459		N
36	FAT	MILK	VOLU	3.229	PCT.	1.55	3.44	0/ 6	AN04C	<16.055	70 > .MITCHELL.50,..537.(1967).	1.0259		*
37	FAT	MILK	VOLU	3.239	PCT.	0.89	2.82	0/ 6	AN01C	<16.055	70 > .MITCHELL.50,..537.(1967).	0.8414		*
38	FAT	MILK	VOLU	3.530	PCT.	1.70	3.02	0/ 6	AN05C	<16.055	70 > .MITCHELL.50,..537.(1967).	0.9128		*
39	FAT	MILK	VOLU	3.750	PCT.	1.54	2.48	0/ 6	AN07C	<16.055	70 > .MITCHELL.50,..537.(1967).	0.7565		*
40	FAT	MILK	VOLU	3.779	PCT.	1.15	1.97	0/ 6	AN03C	<16.055	70 > .MITCHELL.50,..537.(1967).	0.6016		*
41	FAT	MILK	VOLU	3.875	PCT.	1.32	2.45	0/ 6	AN02C	<16.055	70 > .MITCHELL.50,..537.(1967).	0.7510		*
42	FAT	MILK-CHO	VOLU	1.851	PCT.	0.95	3.94	1/ 5	AN11C	<16.055	70 > .MITCHELL.50,..537.(1967).	1.0807		*
43	FAT	MILK-CHO	VOLU	1.867	PCT.	3.54	5.52	0/ 6	AN10C	<16.055	70 > .MITCHELL.50,..537.(1967).	1.5160		*
44	FAT	MILK-CHO	VOLU	3.168	PCT.	0.53	2.21	0/ 6	AN12C	<16.055	70 > .MITCHELL.50,..537.(1967).	0.6572		*
45	FAT	MILK-CHO	VOLU	3.188	PCT.	1.88	3.01	0/ 6	AN09C	<16.055	70 > .MITCHELL.50,..537.(1967).	0.8959		*
46	FAT	MILK-DRI	GRAV	0.912	PCT.	4.46	7.17	0/ 5	BL03B	<16.219	84 > .KEISTER.10 0308(1927).	1.7679		I
47	FAT	MILK-SKI	INFR	0.053	PCT.	80.28	169.62	0/ 6	AC27FK	<OMIT-SKIM>.	.BIGGS.61,..015.(1978).	27.2779		N
48	FAT	MILK-SKI	INFR	0.055	PCT.	80.29	176.98	0/ 6	AC18FK	<OMIT-SKIM>.	.BIGGS.61,..015.(1978).	28.5937		N
49	FAT	MILK-SKI	INFR	0.068	PCT.	71.44	121.90	0/ 6	AC24FK	<OMIT-SKIM>.	.BIGGS.61,..015.(1978).	20.3488		N
50	FAT	MILK-SKI	INFR	0.072	PCT.	46.94	118.71	0/ 6	AC27FG	<OMIT-SKIM>.	.BIGGS.61,..015.(1978).	19.9936		N
51	FAT	MILK-SKI	INFR	0.074	PCT.	60.67	122.89	0/ 6	AC24FG	<OMIT-SKIM>.	.BIGGS.61,..015.(1978).	20.7686		N
52	FAT	MILK-SKI	INFR	0.081	PCT.	36.24	83.57	0/ 6	AC18FG	<OMIT-SKIM>.	.BIGGS.61,..015.(1978).	14.3076		N
53	FAT	MILK-SKI	INFR	0.113	PCT.	25.98	81.35	0/ 6	AC21FG	<OMIT-SKIM>.	.BIGGS.61,..015.(1978).	14.6543		N
54	FAT	MILK-SKI	INFR	0.120	PCT.	40.99	62.62	0/ 5	AC21FK	<OMIT-SKIM>.	.BIGGS.61,..015.(1978).	11.3777		N
55	MOISTURE	CHEESE-B	GRAV	41.198	PCT.	0.85	3.59	1/11	BE03AV	<16.259	84 > .STRANGE.52 .117.(1969).	1.5707		N
56	MOISTURE	CHEESE-B	GRAV	41.478	PCT.	0.38	3.61	0/11	BE03BV	<16.259	84 > .STRANGE.52 .117.(1969).	1.5811		N
57	MOISTURE	CHEESE-R	GRAV	45.894	PCT.	0.45	3.31	1/11	BE04AV	<16.259	84 > .STRANGE.52 .117.(1969).	1.4719		N
58	PROTEIN	BUTTERFA	COLO	3.222	PCT.	.	1.74	0/ 5	AJ12B	<16.037	84 > .SHERBON.58 .773.(1975).	0.5188		
59	PROTEIN	BUTTERFA	COLO	3.303	PCT.	.	8.42	0/ 5	AJ12A	<16.037	84 > .SHERBON.58 .773.(1975).	2.5197		
60	PROTEIN	MILK	INFR	2.953	PCT.	1.21	1.99	0/ 6	AC01PA	<16.083	84 > .BIGGS.61,..015.(1978).	0.5856		S
61	PROTEIN	MILK	INFR	2.966	PCT.	0.47	2.15	0/ 6	AC04PA	<16.083	84 > .BIGGS.61,..015.(1978).	0.6331		S
62	PROTEIN	MILK	INFR	2.979	PCT.	0.42	2.41	0/ 6	AC02PA	<16.083	84 > .BIGGS.61,..015.(1978).	0.7101		S
63	PROTEIN	MILK	INFR	3.100	PCT.	0.60	2.27	0/ 6	AC03PA	<16.083	84 > .BIGGS.61,..015.(1978).	0.6729		S
64	PROTEIN	MILK	INFR	3.109	PCT.	0.31	2.50	0/ 6	AC05PA	<16.083	84 > .BIGGS.61,..015.(1978).	0.7414		S
65	PROTEIN	MILK	INFR	3.284	PCT.	0.40	2.11	0/ 6	AC06PA	<16.083	84 > .BIGGS.61,..015.(1978).	0.6309		S
66	PROTEIN	MILK	INFR	3.603	PCT.	0.59	2.91	0/ 6	AC07PA	<16.083	84 > .BIGGS.61,..015.(1978).	0.8823		S
67	PROTEIN	MILK	INFR	3.634	PCT.	0.21	2.70	0/ 6	AC08PA	<16.083	84 > .BIGGS.61,..015.(1978).	0.8197		S
68	PROTEIN	MILK	VOLU	0.380	PCT.	1.32	2.62	0/16	AP05A	<16.036	84 > .STRANG.52,.126.(1969).	0.5662		P
69	PROTEIN	MILK	VOLU	0.391	PCT.	1.55	3.07	1/16	AP09A	<16.036	84 > .STRANG.52,.126.(1969).	0.6663		P
70	PROTEIN	MILK	VOLU	0.393	PCT.	1.35	2.90	0/16	AP11A	<16.036	84 > .STRANG.52,.126.(1969).	0.6300		P
71	PROTEIN	MILK	VOLU	0.405	PCT.	1.56	2.76	1/16	AP01A	<16.036	84 > .STRANG.52,.126.(1969).	0.6023		P
72	PROTEIN	MILK	VOLU	0.426	PCT.	1.66	3.12	0/16	AP12A	<16.036	84 > .STRANG.52,.126.(1969).	0.6860		P
73	PROTEIN	MILK	VOLU	0.468	PCT.	1.60	2.60	0/16	AP03A	<16.036	84 > .STRANG.52,.126.(1969).	0.5798		P
74	PROTEIN	MILK	VOLU	0.487	PCT.	0.81	2.26	0/16	AP10A	<16.036	84 > .STRANG.52,.126.(1969).	0.5070		P
75	PROTEIN	MILK	VOLU	0.488	PCT.	1.02	2.38	0/16	AP02A	<16.036	84 > .STRANG.52,.126.(1969).	0.5341		P
76	PROTEIN	MILK	VOLU	0.491	PCT.	1.19	2.69	0/16	AP04A	<16.036	84 > .STRANG.52,.126.(1969).	0.6042		P
77	PROTEIN	MILK	VOLU	0.496	PCT.	1.07	2.25	0/16	AP06A	<16.036	84 > .STRANG.52,.126.(1969).	0.5062		P
78	PROTEIN	MILK	VOLU	0.499	PCT.	1.12	3.14	0/16	AP07A	<16.036	84 > .STRANG.52,.126.(1969).	0.7071		P
79	PROTEIN	MILK	VOLU	0.531	PCT.	0.60	2.09	1/16	AP08A	<16.036	84 > .STRANG.52,.126.(1969).	0.4750		P
80	PROTEIN	VEGTABLE	COLO	1.860	PCT.	.	0.99	0/ 5	AJ13A	<16.037	84 > .SHERBON.58,.773.(1975).	0.2717		
81	PROTEIN	VEGTABLE	COLO	1.888	PCT.	.	4.03	0/ 5	AJ13B	<16.037	84 > .SHERBON.58,.773.(1975).	1.1087		
82	PROTEIN	WHIPCREA	COLO	1.744	PCT.	.	4.48	0/ 5	AJ11A	<16.037	84 > .SHERBON.58,.773.(1975).	1.2178		
83	PROTEIN	WHIPCREA	COLO	1.748	PCT.	.	3.72	0/ 5	AJ11B	<16.037	84 > .SHERBON.58,.773.(1975).	1.0115		
84	SALT	BUTTER	VOLU	0.021	PCT.	30.55	59.16	0/ 7	AW6A	<15.135	65 > .WEIK.49,.518.(1966).	8.2939		N
85	SALT	BUTTER	VOLU	0.027	PCT.	24.12	43.11	0/ 7	AW6B	<16.235	84 > .WEIK.49,.518.(1966).	6.2627		N
86	SALT	BUTTER	VOLU	0.989	PCT.	4.58	6.23	0/ 7	AW2A	<15.135	65 > .WEIK.49,.518.(1966).	1.5550		
87	SALT	BUTTER	VOLU	1.287	PCT.	1.31	4.47	0/ 7	AW5A	<15.135	65 > .WEIK.49,.518.(1966).	1.1608		
88	SALT	BUTTER	VOLU	1.535	PCT.	0.46	4.23	1/ 7	AW7A	<15.135	65 > .WEIK.49,.518.(1966).	1.1280		
89	SALT	BUTTER	VOLU	2.090	PCT.	2.20	3.44	0/ 7	AW1A	<15.135	65 > .WEIK.49,.518.(1966).	0.9609		
90	SALT	BUTTER	VOLU	2.256	PCT.	4.55	6.38	0/ 7	AW3A	<15.135	65 > .WEIK.49,.518.(1966).	1.8027		
91	SALT	BUTTER	VOLU	2.386	PCT.	0.50	5.75	1/ 7	AW4A	<15.135	65 > .WEIK.49,.518.(1966).	1.6385		

example, if 100 RSD_R values are given, the y-values would range from 0 to 100% and the x-axis points would range from the lowest to highest RSD_R values of interest. (In the case of the normal distribution, the resulting cumulative distribution curve is the familiar "S-shaped" curve. When such data are plotted on normal-probability paper, the groups that are nearly normal will give a straight line.) When a number of such curves are placed on the same graph, their characteristics (spreads, slopes, and smoothness) may be compared visually, particularly with respect to slope and overlap, which would qualitatively indicate significance or nonsignificance of the differences in the groups of RSD_R values for the various analyte-matrix-method combinations. Figure 1 gives the cumulative distribution curves for the approved methods for the determination of fat in various milks and milk products as an example. The curve formed by the 12 points marked "D" is interpreted as follows: 50% of the RSD_R values (the y-axis) are at or below an RSD_R value of 3% (x-axis) and 50% are above.

From any cumulative distribution curve, the RSD_R values corresponding to the 25%, 50%, and 75% intervals (quartiles), for example, can be easily read off. In addition, the interpolated empirical values can be read off at the 5% and 95% points to establish empirical 90% limits about the medi-

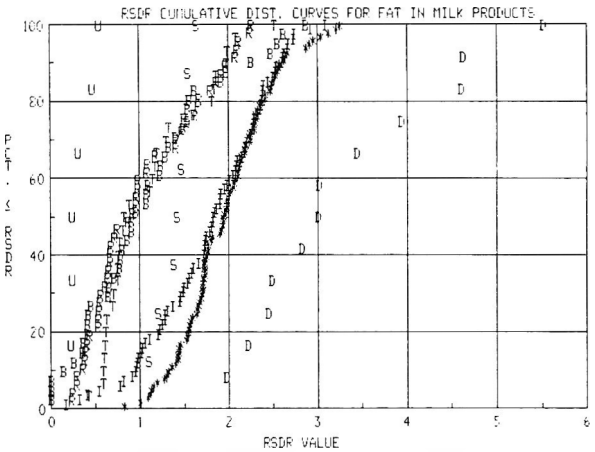

Figure 1. Cumulative distribution curves of RSD_R for available methods for determination of fat in milks and milk products (Table 4). Key to methods: (R) Roese-Gottlieb for milk, chocolate milk, and dried milks; (B) Babcock for milk; (I) infrared for milk; (D) detergent for milk and chocolate milk; (T) automated turbidity for milk; (S) Schmid-Bondzynski for cheese; (U) indirect extraction method for butter; (*) simulated curve from assumptions of RSD_R = 2%, RSD_r = 1%, L = 8, and k = 2. Assays with concentrations of less than 2.5 g fat/100 g have been omitted. (Minor discrepancies among number of assays in various figures and tables result from application of different limitations.)

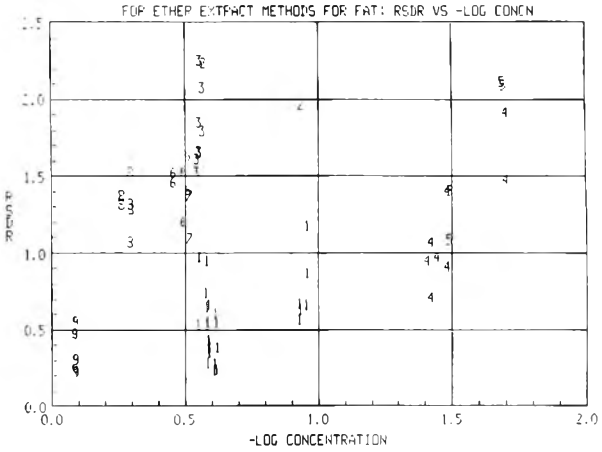

Figure 2. Effect of concentration, expressed as −log C, on RSD_R values for individual assays for determination of fat by ether extraction methods in dried milk (designated 1, 2, 3, 7), homogenized milk (4), chocolate milk (5); cheese (Schmid-Bondzynski) as-is basis (6), dry basis (8); and butter (9).

an for any given set of RSD_R values. This commonly used 90% interval corresponds to an empirical interval within which values for RSD_R occur 90% of the time. This interval provides the baseline for assays already performed. It must be clearly understood that these curves provide a visual presentation of the *distribution* of the set of calculated between-laboratories (including within-) relative standard deviations as found in the literature, rather than just a single "true" estimate of the desired reproducibility value pertaining to a single (or replicate) analytical result.

Cumulative distribution graphs for the RSD_R estimates by analyte-methods across all pertinent studies are given and discussed later in the appropriate sections. As a check on the operation of the program (7), and assuming the following typical collaborative study values: RSD_R = 2%, RSD_r = 1%, L = 8, and k = 2, the curve obtained by computer simulation, marked "*", is also plotted on some graphs. As expected, the 50% point, where half of the RSD_R values should be less than or equal to 2% (and half above), occurs near the (assigned) true value of 2%.

These RSD_R values relate only to the precision estimates, independent of systematic error. Many of the methods of milk analysis (e.g., solids, fat, ash, acidity) are empirical—that is, the results are defined in terms of the method of analysis. In such cases, no allowance is made for systematic error. Moreover, in most cases, systematic error has been taken into consideration in setting the standard or specification which is appraised by the method. Where a standard or specification is established in terms of an empirical reference method (e.g., fat in milk by the Roese-Gottlieb method), secondary methods (e.g., Babcock or infrared methods) must be calibrated by or adjusted to the reference method. Any residual differences between methods are then confounded with the precision estimates of the random error.

Similar statistical analyses and graphs could be presented for RSD_r, but this parameter fluctuates very widely within any given study, although its long-term average seems to be about 0.5–0.7 × RSD_R, and it is always bounded by 0 and the RSD_R of the assay, by definition.

Effect of concentration.—Since the composition of milk products is fairly constant, the effect of analyte concentration (C) on RSD_R usually need not be taken into consideration in presenting the precision parameters for a single product. The intrinsic variability of RSD_R values is considerably greater than could possibly be due to the effect of concentration. The concentration effect may become significant when RSD_R estimates are compared for an analyte (e.g., fat or solids) in a series of substantially different products characterized by substantially different levels of that analyte (e.g., fat in skim milk, whole milk, ice cream, dried milks, cheeses, butter, and anhydrous milk fat). In such cases, the concentration effect can be taken into consideration by using a linear regression of log RSD_R on log C. Another special case involves the Babcock, Gerber, and detergent methods for fat that use volumetric measurements. Here the error of measuring the volume of the fat column in any given type of bottle is constant regardless of the amount of fat. In actual practice, these volumetric methods provide for varying the size of the test portion and the volume of the measuring column when testing skim milks, cream, and ice cream. However, in the referenced collaborative studies, no provision was made for handling this situation; apparently the same test bottles were used for both part-skim and whole milks. Consequently, some of the part-skim milks containing about 2% fat show RSD_R values of about 5%, which is more than double the typical RSD_R value obtained for studies using whole milk with a fat content of about 3.5 g/100 g. The same situation with respect to a constant measurement error probably exists in the measurement of a difference in absorbance values in the infrared method for fat in milk.

A plot of the RSD_R values against $-\log_{10}C$ (C = concentration expressed as a decimal fraction, e.g., 100% = 1.00) for the individual analyte assays shows, in many cases, a slight concentration effect but with a large overlap. For example, Figure 2 is a plot of 63 RSD_R values from studies of ether extraction methods for the determination of fat in various milk products. The RSD_R values for individual assays for fat in dried milks (labeled 1, 2, 3, and 7, from different studies), homogenized milk (labeled 4), and chocolate milk (labeled 5) are plotted against $-\log_{10}C$. The negative logarithmic scale of the fractional concentration is used for 2 reasons: First, this plotting technique has the effect of spreading out the lower concentrations for better resolution of the RSD_R values rather than crowding them together as zero is approached; second, comparability is maintained with

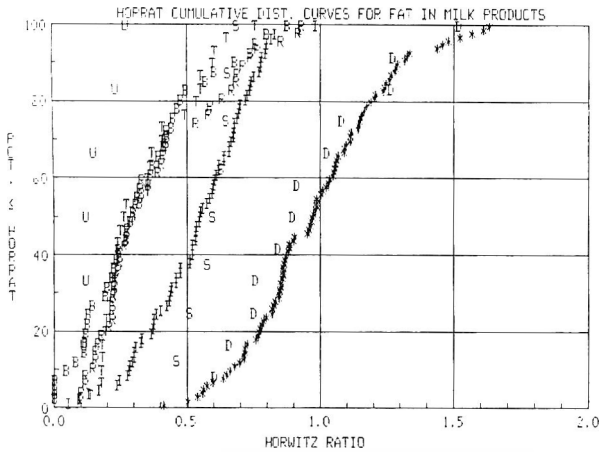

Figure 3. Cumulative distribution curves of RSD_R values shown in Figure 1, plotted as function of ratio of RSD_R found to RSD_R calculated from Horwitz equation: RSD_R (%) = 2 exp (1 − 0.5 $\log_{10}$C), where C is concentration expressed as decimal fraction.

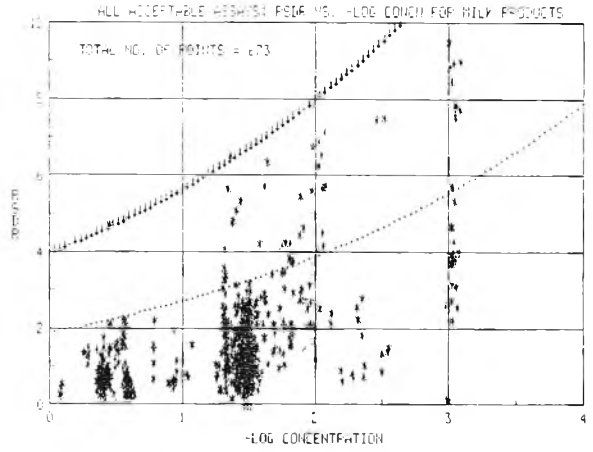

Figure 4. RSD_R values for all 661 acceptable assays as function of $-\log_{10}$C, where C is expressed as decimal fraction. Lower line is Horwitz equation, RSD_R = 2 exp (1 − 0.5 $\log_{10}$C); upper line is twice this curve and is considered upper empirical limit for all analytes, independent of matrix and method.

other papers in this series. In addition, the RSD_R values are plotted for assays by the ether extraction method for fat in cheese, on an as-is basis (labeled 6) and for the same cheeses on a dry basis (labeled 8), and for assays by the indirect method for fat in butter (labeled 9) in which the fat is removed by petroleum ether extraction. Cheese and butter are distinctly different food matrixes with considerably different fat concentrations, but the RSD_R values for the assays for fat of these products overlap those for the assays of other milk products. Expression of the analyte on the "as-is" or "dry" basis has little effect on RSD_R since the effective concentrations change little, from 30 to 50 g/100 g.

In Figure 2 the RSD_R values cluster by product and by study within products. Although it is tempting to eliminate the studies (1932)0524PAZ and (1927)0308PBL on dried milks (labeled 2 and 3) because of their age, this cannot be justified since there has not been any basic change in the Roese-Gottlieb ether extraction method since its inception. But with or without these clusters, the variances among the RSD_R estimates from the dried and fluid milks are unequal. The linear regression has heterogeneous variances and a low correlation coefficient (r = 0.35). At best the regression indicates a trend of decreasing RSD_R with increasing concentration, but the wide spread of RSD_R values within each cluster suggests that the use of predictive equations for RSD_R from g fat/100 g would be of questionable value. The same situation exists for other analytes, when sufficient data exist for a similar visual examination. For most purposes RSD_R can be assumed to be constant over a moderate concentration range, i.e., approximately one order of magnitude, except when the measurement error is approximately constant and the relative error therefore becomes greater the lower the concentration, as in the case of volumetric and infrared methods.

The fact that the RSD_R values are independent of concentration for single or related products over a restricted concentration range does not conflict with the use of the general Horwitz equation (10) for the calculation of RSD_R values which applies when a range of concentrations over several orders of magnitude is involved.

Summaries.—The final form for presentation of the "raw" data ($\bar{x}$, RSD_r, and RSD_R) for each analyte-method-product (e.g., protein by the Kjeldahl method in liquid whole milk) collaborative assay and for each analyte over all methods in all products (e.g., all protein values) is a cumulative distribution curve of RSD_R that ignores the analyte concentration. When RSD_R varies significantly with concentration, the ratio of RSD_R found to RSD_R predicted by the Horwitz equation (designated "HORRAT") (10) is used. This transformation substantially removes the concentration effect when several concentration levels are involved. A HORRAT of 1.0 indicates that the line of best fit, when HORRAT is plotted against concentration or log concentration, follows the Horwitz curve exactly for a set of assays; higher values are above the curve and lower values are below it. The data from Figure 1 were transformed in this manner and plotted in Figure 3. The shapes and slopes of the curves on the ratio scale are very similar to those on the linear RSD_R scale, but the relative positions of the curves are shifted somewhat.

Cumulative curves as well as histograms of frequency vs RSD_R and frequency vs HORRAT were also prepared for each important analyte-method-matrix combination. Such arrays permit comparisons of direct precision data and "normalized" (with respect to concentration) precision data across products and methods. In Figure 4, a graphical summary is presented of the individual RSD_R values from all the 661 accepted assays for milk products as a function of $-\log_{10}$C for comparison with the corresponding graphs for other commodities in other papers, as for example in drugs (10). A tabular summary of the precision parameter RSD_R and its dispersion is given in Table 2 for use with the referenced compilations (1, 4).

The cumulative distribution curves are interpreted in terms of the 90% intervals. In Figure 1, as an example, the 90% interval for fat by the Roese-Gottlieb method (labeled R) would be between 0.3 and 2, to 1 significant figure. It was observed that 90% of RSD_R values (based on the empirical frequency distribution) from assays of milks for fat by the Roese-Gottlieb method were found within the interval 0.3–2%. Although most values were near the median, 1%, an occasional extreme value of 0.2 or 2.3% was observed. The 90% interval cannot be used to predict future results. However, the data presented in the tables should provide a useful reference for discussion of precision estimates obtained in the future. Because of this intrinsic variability, the final precision parameters regardless of their nature (S, R, or RSD) should be given to only 1 significant figure. More significant figures may be used in the tables prepared to determine if significant differences exist among groups, as in Table 2.

Use of the reproducibility value, R, or other functions.—The cumulative distribution curves of R also may be plotted,

Table 2. Summary table of median and 90% interval of between-laboratories relative standard deviation precision parameter, RSD_R, for determination of various constituents in milk and milk products, pooling all methods[a]

Constituent	No. of assays	Concn, g/100 g	RSD_R, % Median	RSD_R, % 90% limits[b]
Fat	214	2.5–80	1.3	0.3–2.6
Protein	201	3–40	1.1	0.4–2.6
Solids	30	10–30	0.6	0.2–1.0
Moisture	58	16–40	0.7	0.4–1.5
Major constituents	509	2.5–80	1	0.3–3
Phosphorus	58	0.1–1	4	2–9[c]
Calcium	6	1	3	2–4
Potassium	6	1.5	4	3–5
Sodium	6	0.4	6	5–8
Lactose	27	4–5	2	1–6[c]
Acidity	4	—		
Ash	9	2–3	2	1–4
Salt (butter, cheese)	16	1–2	2	1–3
Freezing point	7	—	0.8	0.6–1

[a] Relative standard deviations are independent of method within stated concentrations. Corresponding standard deviations, S, and reproducibility values, R, must be calculated for specific concentration of analyte of interest. R, % = $0.028 \cdot RSD_R$ (%)$\cdot\bar{x}$ (g/100 g). (Two laboratories should check each other within this amount 19 times in 20.)

[b] 90% limits: RSD_R values lie between these upper and lower limits 90% of the time.

[c] Interval is too broad to be useful.

but only when a single product with a narrow concentration range is involved. These R values are routinely reported in the product-specific methods published by IDF, ISO, and Codex Alimentarius. The R (and r) values as point estimates are *specifically dependent on concentration*, having the same units as standard deviations, and therefore usually apply only to individual products. This is an advantage as well as a disadvantage; R values cannot be extrapolated across products with substantially different levels of an analyte. For concentration ranges over which R values may be usefully extrapolated, the R interval is undoubtedly more narrow than those for the RSD_R interval. An average RSD_R value of 1% for the determination of fat in dried milks corresponds to an absolute content of 0.25 g fat/100 g for a dried milk containing 25 g fat/100 g; the upper 90% limit of RSD_R values of 2% corresponds to 0.5 g fat/100 g. However, an occasional RSD_R value corresponding to 0.05 g fat/100 g or as high as 0.55 g fat/100 g may also be expected.

These RSD_R values for fat are the exact equivalent of the R values at the same concentration. The relationship is $R = 0.028 \cdot RSD_R \cdot \bar{x}$ (5). The equivalent R values for dried milk containing 25 g fat/100 g are 0.21 g fat/100 g for the lower RSD_R prediction limit of 0.3% and 1.4 g fat/100 g for the upper prediction limit of 2%, if RSD_R is expressed as a percentage and $\bar{x}$ as g/100 g. These values can be compared with the R value of 0.3 g fat/100 g given in IDF method 9C:1987. The R value for a median RSD_R value of about 1% would be 0.70 g fat/100 g. However, occasionally this limit, calculated from an individual collaborative assay, can be as small as 0.1 or as large as 1.5 g/100 g at the same analyte concentration. The uncertainty problem that exists for these reproducibility values is the same that exists for the standard deviations and relative standard deviations.

Individual Analytes by Methods

The studies of the analytes of interest for the present review were those referenced in SMEDP and any others encountered during the course of browsing through the referenced volumes. The following sections discuss the precision characteristics of the studies for the individual analytes, matrixes, and methods. Because of the paucity of collaborative studies for many analyte-matrix-method combinations, combinations with similar RSD_R values and reasonable matrix relationships were merged as far as possible.

Fat

Fat, Roese-Gottlieb method.—The Roese-Gottlieb (or its equivalent) ether extraction method for the determination of fat in milk, 16.064[84], and in dried milk, 16.219[84], is the reference method used to determine fat in most milk products by many methods-endorsing and standards-setting organizations. It is used for calibrating instrument methods, since as the reference method it has no systematic error, by definition. The fat is liberated by addition of ammonium hydroxide, extracted in the presence of ethanol with ether-petroleum ether, and weighed after removal of the solvent by evaporation.

Nine independent collaborative studies of ether extraction methods were available on milks: 7 on dried milks (11–26 g fat/100 g) and 2 on fluid milk, including chocolate milk. Five independent IDF studies on dried milks, which explored minor modifications with respect to the use of ethanol, were recently published in a single report, *IDF Bulletin* 235(1988). Despite the potential source of variability, these assays showed the smallest RSD_R values and least variability among the studies, 0.5–0.8%; the first 2 AOAC studies showed the highest variability, 1.9%. The RSD_R values summarized by studies and products are given in Table 3. Data on fat in cheese and butter by ether extraction methods that have RSD_R values similar to those from the methods for fat in milk are given in the same table and are discussed later.

To determine if a product (matrix) effect is present, the RSD_R values from 35 dried milks assays (symbol D) and the 12 fluid milk assays (symbol F) by the Roese-Gottlieb method were plotted separately by cumulative frequency in Figure 5. The RSD_R values from both sets begin and end at roughly the same regions of the plot although they take different paths, which may merely reflect the disproportionate number of assays in the 2 sets of data. The data from the 2 sets were, therefore, combined into a single curve (symbol M) in Figure 5. The resulting cumulative curve of the 47 individual collaborative assays approximates a straight line with a range of RSD_R from 0.2 to 2.3%. The extremes differ by a factor of about 10. The 90% interval is 0.3–2.1%. The changes in the slope of the curve often indicate breaks between studies, further suggesting within-study correlation. A matrix effect may be present with lower RSD_R values tending to be produced with higher fat products and higher RSD_R values produced by part-skim dried milks and chocolate milks. However, many additional independent studies of these products would be required to establish if the matrix and concentration effects are statistically significant. Particularly unfortunate is the lack of assays of part-skim (2 g fat/100 g) fluid milks, which by extending the concentration range of fat by a factor of 2 may permit the isolation of a matrix effect. In the absence of such studies, however, the best that can be concluded is that if a matrix effect exists for fat in the various types of milk, it would appear to consist of minor perturbations of an overriding concentration dependence.

The values of the within-laboratory relative standard deviations, RSD_r, of the individual assays summarized in Table 3 vary from 0.04 to 2.1%. The extremes differ by a factor of 50, but of course over a range of only 2.0% in magnitude. On a relative basis, this spread is considerably greater than the

Table 3. Summary of size and statistical parameters of individual collaborative studies of determination of fat by ether extraction methods for milk, 16.064[84], dried milks, 16.219[84] (SME 18.8.E), cheese 16.284[84], and butter 16.232[84]

Milk product	Study ref.	N	L	k	D	Mean, % fat	RSD_r, %	RSD_R, %
Dried	IDFBUL235,0042(1988)	5	5	2	50	26.4 ± 0.8	0.6 ± 0.3	0.8 ± 0.2
	PBI	5	5	2	50	26.5 ± 1.0	0.2 ± 0.1	0.5 ± 0.2
		5	4	2	40	—	0.4 ± 0.2	0.6 ± 0.3
		5	4	2	40	—	0.3 ± 0.1	0.5 ± 0.3
		5	4	2	40	—	0.3 ± 0.2	0.5 ± 0.2
	(1932)0524PAZ	8	7	2	112	27.9 ± 0.6	0.5 ± 0.2	1.8 ± 0.2
	(1927)0308PBL	2	5	2	24	—	0.2	2.1
Homogenized	(1967)0537PAN	8	6	2	96	3.2 ± 0.8	0.6 ± 0.6	1.2 ± 0.4
Chocolate	(1967)0537PAN	4	6	2	48	2.6 ± 0.7	1.3 ± 0.8	1.7 ± 0.5
Cheese	(1948)0300PAV)	4	5	2	40	32.7	0.6 ± 0.1	1.0 ± 0.4
	(1949)0303PAV)	4	5	2	40	32.6	0.5 ± 0.2	1.5 ± 0.1
	(1933)0584PBC	2	10	1	20	30.3	0.4	1.2
Butter	(1952)0194PBD	6	6	9	324	81.3 ± 0.1	0.06 ± 0.02	0.4 ± 0.1
Total or average		63	6	2	924			

Abbreviations: N = number of assays; L = number of laboratories; k = number of replicates; D = number of determinations (= NLk); — = not meaningful (both whole and part-skim milks present); ± prefixes the "dispersion" standard deviation (not percentiles);) indicates that the same cheeses were used in the 2 studies; the group of 3 letters beginning with P is the key to computer retrieval of the raw data for the cross-referencing.

corresponding spread for RSD_R. The larger spread of within-laboratory variability (greater variability), when it is expected to be smaller, is typical of all method-matrix-level assays and, therefore, this parameter was not explored further with any analyte. In any case, for practical quality control, RSD_r (or r) must be redetermined frequently by every laboratory.

The RSD_R values transformed to ratios of RSD_R values found to RSD_R values predicted by the Horwitz equation (HORRAT) for the ether extraction methods for fat are also plotted as cumulative distribution curves in Figure 6. This transformation, to a great extent, removes the effect of concentration on RSD_R. Such a HORRAT plot is only slightly steeper and the gaps are somewhat more pronounced than the direct RSD_R plot, but no new features are apparent.

If HORRAT is plotted against −log concentration for a series of assays, a horizontal line at 1.0 on the graph would indicate that all assays follow the Horwitz curve. The important aspect of such a plot, which is not shown, is that at worst the gravimetric method for the determination of fat in milk products is considerably more precise by a factor of about 0.5 than would have been predicted from the Horwitz curve, which was derived from numerous commodity areas, containing many different analytes over a range of concentration of 10^9, analyzed primarily by instrumental methods. This was also the case for the gravimetric and titrimetric methods used for the examination of drug products in approximately the same concentration range, 1–100 g/100 g (10).

Overall, when all the available assays are examined together, it is obvious that the precision parameters for the determination of fat in milks by the Roese-Gottlieb method cannot be given by a single value even if the difference in analyte concentration between dried and fluid milk is taken into consideration. The median RSD_R value is about 1% and the 90% empirical interval is about 0.3–2%, based on pooled RSD_R estimates across all assays, regardless of study. To obtain the 90% empirical interval (5% to 95%), a plausible guide to future assays, for the corresponding ISO reproducibility values, R, each R value must be calculated individually: Multiply each RSD_R value, in %, by 0.028 and by its appropriate concentration, $\bar{x}$, in g/100 g:

$$R = 0.028 \cdot \bar{x} \cdot RSD_R$$

Then these R values must be re-sorted to locate the 90% limits. The calculated R values for the entire set of RSD_R values are separated for the fluid and dried milk to obtain the

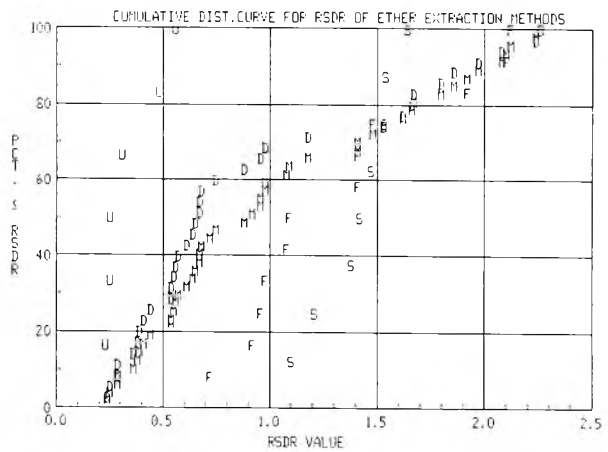

Figure 5. Cumulative distribution curves for ether extraction methods as applied to various products: butter (indirect method) (labeled U); dried milks (labeled D); fluid milks (labeled F); combined dried and fluid milks (labeled M); and cheese (labeled C).

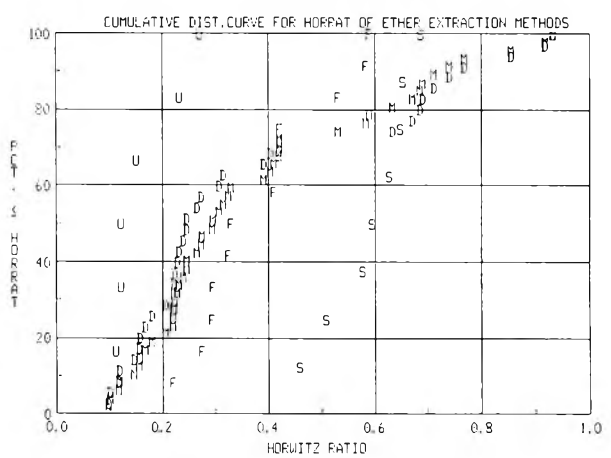

Figure 6. Data of Figure 5 plotted as ratios (HORRATs) of RSD_R values found to RSD_R values calculated from Horwitz equation, as in Figure 3.

corresponding median and 90% limits, which are given below for comparison with the IDF accepted values.

	Precision parameters for fat by Roese-Gottlieb method calculated from:		
RSD_R, %	Median 1	90% interval 0.3–2	R (IDF)
R, milk 3 g/100 g	0.084 g/100 g	0.025–0.17 g/100 g	0.04 g/100 g
R, milk, dried, 25 g/100 g	0.70	0.21–1.4	0.3

For a fluid milk containing 3 g fat/100 g, 90% of future *individual analyses* for fat by the Roese-Gottlieb method in one laboratory would be expected to check another single analysis from another laboratory within the range 0.02–0.2 g fat/100 g (to 1 significant figure). The R value given in IDF Method 1C:1987 for raw and processed milk is 0.04 g/100 g, which is equivalent to an RSD_R of 0.48%. The tabulated values are based on the overall cumulative distribution curve for both milks, dried and fluid. If only the fluid milk curve of Figure 5 is used, none of the AOAC collaborative study assays approach an RSD_R value of 0.48%; the lowest AOAC assay RSD_R is 0.7%. Such a large discrepancy between the precision estimates for the Roese-Gottlieb method applied to fluid milk should be resolved, although even the higher value is still an acceptable precision for the conduct of analyses. For fat in dried milks, the RSD_R value from the early study of the AOAC method appears to be much too high, but a new study should be performed to justify removing it from the database of precision estimates.

Butter analysis.—In most countries, the composition of butter is controlled by a limit on the moisture content. In the United States, a congressional standard applies a minimum fat content of 80% fat, "all tolerances allowed for" (11). In a remarkable single study of the preparation of the test sample of butter, (1952)0194PBD, the RSD_R of the 3 reportable constituents of butter, in g/100 g, for 6 assays were for L = 6, k = 9: moisture, 1.4 ± 0.5; (total) solids-not-fat, 2.5 ± 0.5; and fat, 0.4 ± 0.1. For comparison, the IDF R values for water and (milk) solids-not-fat, transformed to RSD_R values, are given below.

	IDF values			Assay values
	R, g/100 g	$\bar{x}$, g/100 g	RSD_R, %	RSD_R, %
Milk solids-not-fat	0.18	1.5	4.3	2.5 ± 0.5
Water	0.16	16	0.36	1.4 ± 0.5
Fat (calculated)	0.24	80	0.11	0.4 ± 0.1

The R of fat (measured by difference) was calculated by the propagation of error theorem, $R = (R_1^2 + R_2^2)^{1/2}$. The few available assays for butter are plotted in the figures with the symbol U.

Here again, a substantial difference exists between the RSD_R value calculated from the IDF standard and the average RSD_R values from the AOAC study. The difference, although it appears large in absolute terms, is probably of no practical significance. The IDF R values are supported by the extensive quality control parameters developed by the Netherlands dairy control authorities from routine monthly analyses of blind control samples submitted over 19 years to their 5–7 official laboratories (12).

Fat, Schmid-Bondzynski method.—This method is essentially the same as the Roese-Gottlieb method except that it is used for products that cannot be dispersed with ammonium hydroxide. Boiling with hydrochloric acid is required to liberate the fat from ripened and hard cheeses, and the digest must be transferred quantitatively to the extraction flask, an operation that requires excellent technique. Only 8 assays from 3 studies were available to characterize the RSD_R values, which are labeled in the figures as "S." The CDF for RSD_R appears to roughly parallel that for the Roese-Gottlieb method, but the parameters are somewhat higher: median, 1.4%; 90% interval, 1.0–1.6%, possibly reflecting some sampling error with cheese as well as the additional analytical manipulations required with this product. Two of the studies performed a year apart, (1948)0300PAV and (1949)0303PAV, used the same cheeses. The average RSD_R values were 1.0 and 1.5%, respectively, reflecting the inherent natural variability (analytical noise) of the estimates of the RSD_R values.

Casein products.—IDF developed a method and conducted the collaborative study for the determination of fat in casein and casein products, IDF235,0067(1988)PAV. The method is based on the Schmid-Bondzynski ether extraction method for cheese. In these products fat is an undesirable impurity. At the low levels of 1% and below, the RSD_R values range from 4 to 14%, possibly due to heterogeneity of the commodity, use of small (gram) weights of test portion, the presence of a small fractional concentration (0.01) in the denominator, and the typical degeneration of precision with decreasing concentration. A similar deterioration of precision when impurities are determined has been noted in the review of the precision parameters of analytical methods for pesticide formulations (13). Since the impurity category of components constitutes a different population from the composition category, it was not included in the statistical calculations. It is included in the database of rejected assays with a label "I" (for impurity analysis) in the "STAR" column. Fat in nonfat dry milk similarly is an undesired "impurity." A single assay, (1927)0308PBL03B, from this matrix was also placed in the same database for the same reason.

Fat, Babcock method.—Four studies, containing a total of 41 assays, were available for estimating the precision of this method. The assays are labeled "B" in Figures 1 and 3. The 2 cumulative frequency plots, RSD_R direct and HORRAT, are almost identical. The plot of the RSD_R values for the Babcock method is only slightly shallower than the plot of the RSD_R values for the Roese-Gottlieb method, but it has a longer tail at the upper values of RSD_R. These high RSD_R values are from a single study in which all the RSD_R high values (average of 8 assays is 2.9%), including the highest RSD_R values of 4.2 and 5.4%, are from analyses of 2.0 g fat/100 g part-skim milks. So few part-skim milks were included in these studies of milk methods that it appears reasonable to remove these assays from the whole milk population, and to qualify the precision parameters as not being applicable to milks with a fat content less than 2.5%, which approximates the lowest member of the remaining population. A special study on part-skim and skim milks is needed to determine whether the precision results are related to laboratories or to fat content variables. On the basis of the present data from 4 studies and 41 assays, the median RSD_R value of all assays

by the Babcock method is 1.0% and the 90% interval is 0.0–2.5%, equivalent to R values of 0.0–0.21 g fat/100 g for 3 g/100 g milk. These values are comparable to the corresponding values of 0.02 and 0.17 g fat/100 g of 3 g/100 g milk for the Roese-Gottlieb method. The corresponding HORRAT values are median, 0.3, and 90% interval, 0.0–0.7, which are quite acceptable.

These precision parameters do not permit an estimation of any residual systematic errors between the Babcock method and the Roese-Gottlieb reference method. The method variables—strength and amount of sulfuric acid, digestion time and temperature, and conditions of reading the fat column—presumably have been adjusted during the method development work to make the Babcock method give readings equivalent to those by the Roese-Gottlieb method.

Fat, infrared method.—Although only 2 collaborative studies were found for the infrared method, 82 assays were available for calculation of the 90% interval. Replication was performed on 2 days with various types of calibration standards and on various types of milk. The precision of the infrared method for fat begins to degenerate with part-skim milks (less than about 2% fat), and the method is definitely not applicable to skim milks (less than 0.1 g fat/100 g milk), which produced RSD_R values of the magnitude of 50 and 100%. Although the relative difference between 0.02 and 0.03 g fat/100 g is 50%, the absolute difference of 0.01 g fat/100 g is of no practical significance, as with the Babcock method. There is no statement in the AOAC method, 16.083[84], restricting the method to whole milk. However, the IDF draft guide for the determination of constituents by infrared instruments (14) indicates that the milks for calibration should contain "between about 2.5 and 5.0 g of fat per 100 g of milk," which suggests that the method is not applicable to fat contents below 2.5 g/100 g. For this review of precision parameters of the infrared method, the milks with a fat content below 2.5 g/100 g were removed from the acceptable assay database and placed in the database of unacceptable assays with the footnote "N" (not applicable).

Precision parameters for the infrared method calculated from the assays using standards prepared by individual laboratories were also removed as nonconforming values, since the RSD_R values were consistently 2–3 times as high as with the other assays. Laboratories introduce their own systematic errors in preparing their standards, and these systematic errors of the individual laboratories manifest themselves as random errors of the group, as pointed out by Currie (15). Therefore, the summary precision parameters for this method are based on the use of various common standards by the participating laboratories. Reference standards constitute a source of between-laboratories error, independent of the method. This potential source of error should be noted in the description of the method.

The cumulative distribution curves for the infrared method, labeled "I" in Figures 1 and 3, are slightly shallower than those for either the Babcock or Roese-Gottlieb method and initially were characterized by a very long tail at the high RSD_R values. By restricting the assays to milks with fat content greater than 2.5 g/100 g and to those studies using common standards, the tail was eliminated. The cumulative distribution plot of HORRAT is almost identical with the direct RSD_R curve.

The median RSD_R value of the infrared method for fat is 1.8%; the 90% empirical prediction interval is 0.6–2.7%, considerably wider than those of the methods previously considered. These parameters are applicable only to whole milk. Several independent collaborative studies should be performed to provide the corresponding parameters for milks with lower fat content.

Table 4. Statistical parameters characterizing collaboratively studied methods of analysis for fat in milks and milk products (fat greater than 2.5%)

Fat method	No. of assays	RSD_R, % Average	Median	90% interval[a]
Roese-Gottlieb	47	1.1	0.9	0.3–2.1
Babcock	41	1.1	1.0	0.0–2.5
[Detergent	12	3.3	3.0	1.9–5.0]
Infrared	82	1.8	1.8	0.6–2.7
Turbidity	30	1.1	0.9	0.5–2.0
Schmid-Bondzynski (cheese)	8	1.4	1.4	1.0–1.6
Indirect (butter)	6	0.3	0.3	0.2–0.5
Total, omit detergent	214	1.4	1.3	0.3–2.6

[a] 5% to 95% interval. The complete curve is given in Figure 1.

Fat, turbidity (automated).—Only one study consisting of 30 assays, with some laboratories using different standards, was available for the automated method for determining fat based upon turbidity. The RSD_R values, labeled T in Figures 1 and 3, correspond closely to those in the plot for the Babcock method. No milks with a fat content below 3% were used so the RSD_R for lower fat milks is not known. As with the infrared method, the RSD_R value with provided standards (0.9 ± 0.2%) is approximately half the RSD_R value with independently prepared standards (1.6 ± 0.7%). However, a conclusion as to whether this effect, although plausible, is real should not be based upon the results of a single study.

Fat, rapid detergent.—This method is now in surplus status as it is probably not being used. When viewed with the other methods for fat in Figures 1 and 3, it is obvious that the precision of this method is the poorest of all. The difference between the RSD_R values of this method and the RSD_R values of the other methods is undoubtedly significant, both practically and statistically. This method, which is not in SMEDP, should be deleted entirely from *Official Methods of Analysis.*

Fat, summary.—Figures 1 and 3 show the dispersion of RSD_R values for the various methods studied. Table 4 summarizes the characteristics of each method and of the fat methods overall.

For the reference methods (Roese-Gottlieb and Schmid-Bondzynski), it can be stated that the RSD_R values from a future collaborative assay will usually occur in the interval 0.3–2%, with a median value of about 1%. The RSD_R of the Babcock and turbidity (automated) methods will almost always be in the range 0–3%. The 0 arises from the fact that the fat column of the Babcock method is usually read to the nearest 0.05 g (mL)/100 g. It is not unusual for all laboratories of a 5-laboratory study to report the same value. The infrared and automated methods require special attention to the accuracy of the calibration standards. To avoid the frequent occurrence of relatively high RSD_R values, simultaneous controls and use of common standards must be an essential part of the instrumental methods. The indirect method for fat in butter has unusually low RSD_R values, based on 6 assays from a single study, largely because of the high fat content which appears in the denominator. The detergent method should be deleted.

Protein

Protein, Kjeldahl.—The Kjeldahl method is the reference

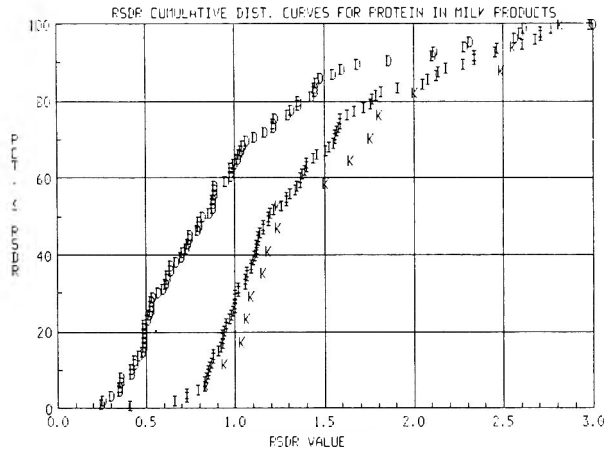

Figure 7. Cumulative distribution curves of RSD_R values for methods for determination of protein in milk products. Key to methods: K = Kjeldahl; I = infrared; D = dye-binding.

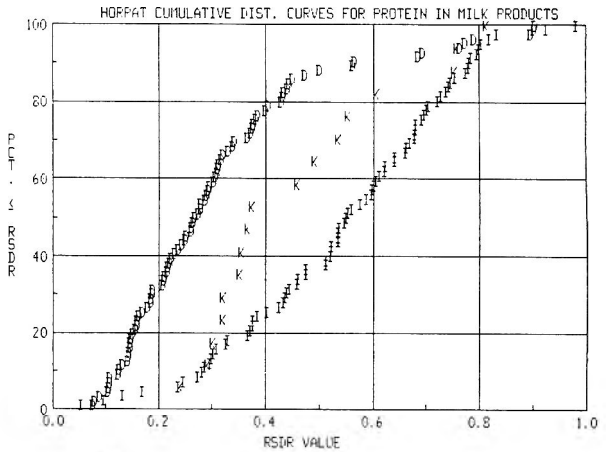

Figure 8. Data of Figure 7 plotted as ratios (HORRATs) of RSD_R values found to RSD_R values calculated from Horwitz equation, as in Figure 3.

method for the determination of protein nitrogen in most commodities. In the series of 4 studies and 34 assays that were available, a number of potentially variable factors are involved—milk and cheese matrixes and mercury and copper catalysts. Each study appears to be independent of the others as shown by the discontinuities in the cumulative graphs (Figures 7 and 8).

The study that produced the highest RSD_R values (2.8 ± 0.5%) for this method, (1969)0126PAP, although entitled a "Collaborative Study," is actually a proficiency study, because the laboratories were permitted to use any variation of the Kjeldahl method and the replicates were performed a month apart on milks preserved with mercuric chloride. This study consisting of 12 assays was removed from the database as not meeting the definition of a collaborative study performed for the purpose of assessing the performance parameters of a specific method of analysis. The method variations may contribute to the between-laboratories random variability.

The 2 other milk studies show RSD_R values of 1.0 ± 0.1% (6 assays) and 2.2 ± 0.7% (10 assays). The latter study was the calibration part of a collaborative study of the infrared method with only 3 or 4 laboratories supplying Kjeldahl data. Only because of the paucity of studies were these data tabulated at all. The remaining milk study, (1988)0893PBJ, was excellent in intralaboratory design (double-split-level) and execution (20 laboratories). The 6 assays provide a very tight group of RSD_R values. This limited range resulted from the incorporation of mandatory internal quality control materials into the method. Laboratories that did not conduct these determinations had poor recoveries and were rejected. The only other study (16) available for comparison was on cheese with a protein content 7 times as high as that of milk. Cheese also presents problems in the preparation of the test sample. Yet the RSD_R value is 1.8 ± 0.6% for 6 assays, which may not be significantly different from the 1% average RSD_R value from the last study on milk. Both values fall within the range of RSD_R values reported for protein (or nitrogen) by the Kjeldahl method for many other commodities (Horwitz, W., & Albert, R., Abstract presented at 1988 AOAC meeting).

Protein dye-binding methods.—The dye-binding method was developed as a very rapid procedure for the determination of protein in milk and milk products. An excess of dye is added to the dispersed product and the amount of dye that does not react with the basic amino acids of the protein is measured. Both acid orange and amido black are permitted dyes but they are not interchangeable and each method must be calibrated in terms of protein determined by the Kjeldahl method. Four studies used acid orange, and 1 used amido black, but the only direct comparison of the 2 dyes was performed in a single laboratory, (1974)1334, and "the [within-laboratory] precision of the method was found to be comparable." However, when the data on a wide variety of milk products are calculated on a comparable basis, the means and "dispersion" standard deviations of the RSD_R values for the acid orange studies are as follows (number of assays in parentheses): 0.8 ± 0.2% (10), 0.5 ± 0.2% (10), 0.9 ± 0.3% (10), 0.5 ± 0.1% (10), 1.1 ± 0.7% (15), 1.0 ± 0.1% (5), 1.6 ± 1.2% (13, omitting a single nonfat dry milk), and 1.8 ± 0.7% (13, omitting a single nonfat dry milk); the value for the one amido black study is 2.7 ± 0.4% (7). There may be a significant difference in precision between the use of the 2 dyes, but the gross imbalance in number of studies and assays precludes a comparison of RSD_R values. Even if the difference in RSD_R values between the 2 dyes is significant, an RSD_R of 2.7% for a 3.2 g protein/100 g product (HORRAT = 0.8) is still an acceptable precision. Only high-fat products, e.g., whipping cream and butterfat mix, show high RSD_R values, suggesting a homogeneity problem.

This data set contains an example of overriding the IUPAC-1987 outlier removal protocol. The range of replicates in a 7-laboratory study of a 35 g protein/100 g nonfat dry milk was 5 g/100 g but an outlier was not flagged because of masking by a second high value. Such a range is technologically impossible in a standardized product such as this so the high value, which was flagged as a straggler, was removed.

Protein, infrared method.—Protein is determined at the same time as the other constituents in the infrared method. In this case there is no problem with skim milks. The calibration problem arising from laboratories preparing separate standards also exists with the protein methods. Assays from studies that did not use a common standard were flagged in the database and were not used in the statistical calculations.

As with fat, only 2 studies of the infrared method for protein were available for review. The first study shows an RSD_R value, 2.2 ± 0.7% (10 assays), of almost twice that of the later one, 1.4 ± 0.5% (88 assays). The studies are 6 years apart so the difference might be attributable to a substantial improvement in instrumental factors. On the other hand, an absolute difference in RSD_R values of 1% between 2 studies is common with other analyte-matrix combinations in other method studies.

Protein, summary.—The cumulative curves, Figures 7

Table 5. Statistical parameters characterizing the collaboratively studied methods of analysis for protein in milk products

Protein method	No. of assays	RSD_R, %		
		Average	Median	90% interval[a]
Kjeldahl	22	1.8	1.6	0.8–2.9
Dye-binding	87	1.0	0.9	0.4–2.5
Infrared	98	1.4	1.2	0.8–2.6
Overall	207	1.3	1.1	0.4–2.6

[a] 5% to 95% interval.

and 8, and the summary data of the individual assays, Table 5, show that the precisions of the infrared and dye-binding methods are comparable, but the precision of the Kjeldahl method is considerably worse by comparison. However, the precision value for the Kjeldahl method is influenced by deficiencies in half of the reported studies. If all of the studies are included, all of the methods show more or less the same 90% interval with respect to RSD_R: 0.5–2.7%. The Kjeldahl method is still the reference method, regardless of its relatively poor precision in 2 of the 4 available studies. The only acceptable milk study showed very good precision probably because the precaution was taken to include internal quality control assays of tryptophan and ammonium oxalate with every batch of analyses, to establish total recovery of the nitrogen at both of the main steps of the method. The single protein in cheese study has comparable parameters.

Lactose

Only one collaborative study for each of 3 methods for the determination of lactose was available. The polarimetric method, which was used for standardizing the infrared method, has the smallest RSD_R, 1.5 ± 0.4%, but only 2 or 3 laboratories were involved in producing this value. The infrared method study is only slightly more substantial with 4 or 5 laboratories participating, providing an RSD_R of 3.0 ± 0.8%. The more recent enzymatic method study had 8 participants, but shows an RSD_R of 5.1 ± 1.0%, with one laboratory exhibiting erratic results. The ratio of the average RSD_R value to that predicted from the Horwitz curve (HORRAT) (10) for the enzymatic method is 1.6, which is high, but still acceptable for general use.

Single studies, particularly with so few laboratories, do not provide a sufficient basis for assignment of precision parameters. All that can be said is that the methods probably have an acceptable precision for general use, but there is insufficient information to establish useful 90% intervals.

Moisture/Solids

A potential problem exists in connection with this category of analyte since some methods and standards are expressed in terms of solids and others in terms of moisture. The 2 analytes in g/100 g are related as

$$[\text{Solids}] = 100 - [\text{Moisture}]; \text{ and}$$

$$RSD_R(\text{Solids}) = [\text{Moisture}] \times RSD_R(\text{Moisture})/[\text{Solids}],$$

where RSD_R(Solids) or RSD_R(Moisture) are the RSD_R values for solids and moisture, respectively, and [Moisture] and [Solids] are the actual analyte concentrations expressed as percents, g/100 g, or as decimal fractions, as long as both values are in the same units. However, the first equation is written specifically for percent or g/100 g; if a decimal fraction is used, the 100 is replaced by 1.00. The transformation is not too important for products such as cheese containing 40 g moisture and 60 g solids in 100 g; it is important for milk with approximately 12% solids. This is not a problem at the present time since most national and international standards for cheese and its products express their composition requirement in terms of maximum moisture, and for milk and milk products in terms of minimum milk solids.

The availability of data for such important characterizing constituents is limited, as shown in Table 6. Although 5 studies were available for the various methods for determining moisture in cheese, the number of cheeses per study varied from 1 to only 6. The comparative collaborative study, using 3 materials, that established the distillation method for blue-mold cheese shows the highest RSD_R value, 2.5%, whereas the oven methods have RSD_R values that are considerably lower. This single study might be considered for rejection as an "outlier." However, the reviews of method studies for other major constituents of milk products show that such extreme values are not unusual.

Overall, the combinations with the largest number of assays, cheese by microwave and by vacuum oven and milks by air and vacuum oven, show a 90% interval for RSD_R for moisture and solids of about 0.3–1%. However, when the distillation method for cheese and the air oven method for

Table 6. Statistical parameters characterizing collaboratively studied methods of analysis for moisture and solids in milk products

Method	Matrix	No. of assays	Average, g/100 g	RSD_R, %		
				Average	Median	90% interval[a]
			Moisture			
Microwave	cheese	18	40	0.7	0.6	0.1–1.0
Vacuum oven	cheese	25	40	0.7	0.7	0.4–1.2
Distillation	cheese	6	40	1.3	1.3	0.2–1.7
Air oven	cheese	3	37	0.8	1.0	—
Air oven	butter	6	16	1.4	1.5	0.4–2.0
Total		58	—	0.8	0.7	0.4–1.5
			Solids			
Air oven	evap milk	6	26	0.6	0.6	0.6–0.8
	milk	6	11	0.6	0.6	0.4–0.8
	cream	6	32	0.4	0.3	0.2–0.8
	evap milk	8	26	0.9	0.8	0.4–1.4
Vacuum oven	evap milk	4	26	0.3	0.3	—
Total		30	—	0.6	0.6	0.2–1.0

[a] 5% to 95% interval.

Table 7. Summary of statistical parameters characterizing collaboratively studied methods of analysis for phosphorus in milk and cheese

Ashing procedure	No. of assays	RSD_R, % Average	Median	90% Interval[a]
IDF Bull. 207 Studies:				
Wet ashing, milk	16	6.2	7.7	1.7–9.1
Dry ashing, milk	16	4.0	3.8	2.5–5.7
Wet ashing, cheese	14	2.7	2.6	1.4–3.9
JAOAC studies, cheese:				
(1985)0102PBM	12	3.4	3.3	2.4–4.4
(1976)1142	[5	7.6]	too few assays	
Overall	58	4.1	3.5	2–9

[a] 5% to 95% interval.

butter, each with only 6 assays, are added, the interval broadens to 0.4–2%.

Elements

Phosphorus.—Some of the largest studies on phosphorus in milk and cheese are confounded with method development and, therefore, are difficult to interpret. The destruction of organic matter by wet ashing and by dry ashing was compared in a milk study conducted by IDF. The results for RSD_R are shown in Table 7.

All of the studies are marred by erratic performance by 2 of the 7–10 participants. Although there was a substantial improvement in precision between the 2 earlier studies and the latest study, designated "wet ashing, cheese," 2 laboratories still had pronounced systematic errors that they were not aware of. This pattern of producing values that are consistently higher and lower than the mean on all test samples can arise only from incorrectly prepared standard solutions. A suggestion was made in the one paper, (1985)0102PBM, that an internal quality control check of systematic error be incorporated by determining the absorbance of a working standard phosphate solution. Such an internal standard might alert outlying laboratories to the possibility that an incorrect standard solution is being used.

The application of the wet ash method to cheese (about 1 g phosphorus/100 g) provides acceptable RSD_R values of a 3% median and a 90% interval of 2–4%. Yet the same method applied to milk (about 0.1 g phosphorus/100 g) provides very poor precision parameters: median, 8%; 90% interval, 2–9%. The dry ash method applied to milk is not much better: median, 4%; 90% interval, 3–6%.

The molybdenum blue spectrophotometric method for the determination of phosphorus is a well-known, standard method for numerous commodities. Yet 4 sets of assays provide average and median RSD_R estimates ranging from 3 to 8% and 90% intervals that are only slightly larger, 1–9%, for concentrations approximating 0.1 and 1% phosphorus. On the basis of these 58 values, it is impossible to provide a useful RSD_R estimate or interval. Analysts using this method should be aware of the possibility of systematic error in their determinations and should conduct determinations of a standard reference material or a standard compound with every new batch of calibration standards and with every series of test samples.

Calcium, potassium, and sodium.—IDF conducted a collaborative study of flame photometry for the determination of alkaline metals in dry milks, using 6 assays for each metal. The precision values for sodium ($RSD_R = 6.5 \pm 0.8\%$) and potassium ($RSD_R = 4.1 \pm 0.8\%$) were considered satisfactory by the reviewers at concentrations of 0.4 and 1.5 g/100 g, respectively. For calcium at 1.0 g/100 g, however, the RSD_R of $7 \pm 1\%$ was considered excessive. The calcium study was repeated using atomic absorption (17) with $RSD_R = 3.1 \pm 0.5\%$ (6 assays), which was considered acceptable. The 90% intervals for these metals are given in Table 2, but it must be kept in mind that these parameters are based on only a single study.

The sodium and potassium assays illustrate the problem of blindly accepting the results of an outlier removal protocol. When outliers are removed automatically from these assays, the resulting 90% RSD_R intervals for each of the 6 assays are 2–8 and 0–5% for sodium and potassium, respectively, instead of the 5–8 and 3–5% intervals calculated without outlier removal. In both cases, the IUPAC-1987 program removed a laboratory from at least one assay and shifted the RSD_R values of one or more assays from the values clustering about the median of the group to a considerably lower 1–2%. The lower values do not seem to be characteristic of the precision parameters of spectroscopic methods at the 1 g/100 g concentration level. Consequently, for these 2 groups of assays, the IUPAC-1987 protocol was overruled, and the precision interval given is that derived from the data without outliers removed.

It should be noted that recent interest in the nutritional aspects of these elements has resulted in the development of reference dried milks, certified for the level of nutritionally important elements. Continuous use of such reference standards in quality control programs would permit the assignment of specific RSD_r values by individual laboratories for their own use, and would make less important the assignment of between-laboratories prediction intervals to these assays. In any case, the availability of only one study does not permit the assignment of a precision distribution for these elements. Since the single study provides HORRAT values of less than 2.0, all that can be really said is that the methods may be assumed to have acceptable precision.

Chloride, salt.—A single reference, (1941)0631PAB, to a study of the standardization of solutions for the determination of chloride shows RSD_R values for the 0.1N silver nitrate standard solution of 0.1–0.2%. Readers may recall that students in elementary quantitative analysis were expected to replicate their results in this standardization exercise to within 0.1%. With the Volhard method, a solution presented as a known shows an RSD_R of 0.1%; when the same method is applied to an unknown salt, however, the RSD_R estimate is 2.3%! Analysts exhibit an unwitting, well-intentioned bias when presented with prior information.

The original AOAC method for salt in butter, 15.135[65], consisted of washing out the salt with numerous aqueous extractions and titrating the washings. This awkward method was replaced by the simple IDF-ISO-AOAC method, 16.235[84], which consisted of diluting the butter with water and titrating the salt directly. The RSD_R was markedly reduced from 5.3 to 2.3% in a single direct comparison of 6 assays by each method. The wet digestion method for salt in cheese, 16.272[84], shows an RSD_R of 2.0% in a single assay with L = 11, k = 3, at 1.4 g NaCl/100 g, (1937)0339PAX. A later comparison of the Volhard and potentiometric methods, (1982)1350PBB, gives $RSD_R = 1.5 \pm 0.4\%$. The study parameters are L = 8 and k = 2. The differences among these values are not meaningful. Combining the 16 assays of salt in butter and cheese at the concentration level of 1–2 g/100 g permits characterizing the salt assays by the Volhard and potentiometric methods with a median RSD_R value of 2% and a 90% RSD_R interval of 1–3%.

Miscellaneous

A number of methods for the determination of constituents of milk products have been accepted on the basis of a single or very few studies. As long as the precision estimates of most of the individual assays are within the RSD_R range of 0.5 to 3% for analyte concentrations between 1 and 100%, the probability is very high that the 90% intervals correspond to those for analytes such as fat and protein, which are supported by over 200 individual collaborative assays for each analyte. However, unless the number of reported assays approaches about 20, the data cannot be considered sufficiently comprehensive for any interpretation other than a statement of whether or not the method is probably acceptable. The methods that fall in this class are discussed below.

Acidity.—Four assays from a single study of evaporated milk in 5 laboratories show an RSD_R range of 0.9–1.5% for 0.3 g lactic acid/100 g. All that can be said with so few assays is that the RSD_R parameter is similar to the RSD_R values for other analytes in milk products.

Ash.—Two studies, each of 4 assays, of evaporated milk in 5 laboratories show the following RSD_R values for an ash content of 1.6 ± 0.1 g/100 g: average, 0.6%; median, 0.5%; and 90% interval, 0.3–1.0%. A single assay of ash in cheese, (1937)0339PAA, L = 11, k = 3, shows an RSD_R of 1.0% at an ash content of 3.4 g/100 g.

Freezing point.—Even the determination of a physical property such as freezing point exhibits an RSD_R of the same magnitude as those for the major compositional components of milk. The 2 available studies with a total of 7 assays provide a tight range of RSD_R values of 0.6–1.1% and a median of 0.8%. The studies are substantial with respect to laboratories (L = 19–20).

Discussion

The data reported here suggest that the procedure of reporting a single precision estimate for methods of analysis should be modified. Examining each collaborative study as it was reported has given rise to the practice of quoting a single value for precision. When studies of similar analytes-methods-matrixes-levels are examined together, it becomes apparent that each study provides an independent estimate of the overall between-laboratories variability, with each study subject to its own idiosyncratic biases and variances. The empirical distribution of the estimates of the compound term RSD_R is unsymmetrical. Such distributions are best described by medians and percentiles, i.e., as values at or below which specified fractions (e.g., quartiles or deciles) of the numbers of reported values occur. Furthermore, precision estimates are functions of concentration, although this relationship is not often apparent or important within the limited concentration ranges typically encountered in the examination of single milk products for composition.

The problem of variability of precision estimates is always present, but is rarely considered in the interpretation of collaborative studies. Mandel and Lashof (18) and Hamaker (personal communication, Feb. 27, 1987) have discussed this problem from a statistical point of view, emphasizing the intrinsic uncertainty in the assessment of variability and its effects on precision estimates. The present paper provides practical examples from interlaboratory studies of the existence of the predicted variability in terms of precision estimates rather than probabilities.

It should be realized, however, that knowledge of the variability in RSD_R estimates is not really what is desired by the analyst. Rarely does an organization anticipate the necessity for an additional collaborative study for the same analyte-matrix-level combination. In fact, the IUPAC effort directed toward harmonization of protocols for collaborative studies (8) was intended to eliminate the need for multiple collaborative studies. Knowledge of the distribution of RSD_R values is useful only to determine if the values from a study are in the acceptable region. On the other hand, what is desired by the analyst is assurance, with a high degree of probability, of being able to attain approximately the same precision in future individual analyses as was attained in the past. This is the function of the ISO reproducibility (R) value (2 laboratories should check each other within this limit 19 times in 20; $R = 0.028 \times RSD_R \times \bar{x}$).

Because the RSD_R values from any given study or combination of studies do not conform to any simple conventional type of distribution, it is necessary to utilize a nonparametric (independent of distribution) function to describe their variability. The empirical limits (ranges, intervals) taken from the cumulative distribution curve are such functions. These limits are defined in the engineering and quality control literature as the boundaries of the interval that will contain 90% (or any other arbitrary percentage) of future values of the quantity or parameters of interest obtained in the same manner. As an example, control charts based on the range can be derived to control current output based on past data (19). The use of cumulative distribution curves for obtaining empirical limits has the advantages of (*1*) ease of direct reading from the cumulative curve, and (*2*) automatic indication of the extreme nontypical values that occur occasionally. These limits are unaffected by small numbers of outliers. However, it must still be kept in mind that the "true" percentile point, in common with all other statistical estimates, is still highly uncertain (18). For example, an empirical 90% interval, in the "true" population, could be as low as an 80% or as high as a 99% interval; the answer depends to a large extent on the number of values available for the estimate.

Sufficient data are available for the between-laboratories relative standard deviation (RSD_R) for fat methods and protein methods to show that their precision estimators are not based on a single underlying population mean, characteristic of the method, but rather that they form an undefinable distribution of values. The values obtained within a single study appear to be characteristic of that study, with only a general relationship to the RSD_R values of another study of the same analyte in the same product at the same level. If the individual assays from all studies of an analyte-method-level combination are plotted together, i.e., RSD_R vs analyte concentration, the resulting distribution is often multimodal, showing correlation of assays within a study, but independence across studies. In the cumulative distribution curve this phenomenon is exhibited as discontinuities in the slope and this may reflect differences in the proficiency of collaborators in different studies. For example, the average values of RSD_R ± the "dispersion" standard deviation for 4 studies of the Babcock test (volumetric-type method) for fat in whole milk range from 0.63 to 2.93% (0.6 ± 0.5, 1.2 ± 0.2, 1.1 ± 0.7, and 2.9 ± 1.2), values that differ by a factor of almost 5 (but an absolute difference of only 2.5%), yet they are supposed to be estimates of the same RSD_R. Had part-skim milks been included, the RSD_R range would have been even larger.

A similar situation exists for protein. The RSD_R estimate for protein in milk by the Kjeldahl method in the original AOAC study is 2.1%, which is almost twice as high as the corresponding parameter estimate from numerous other

studies of the Kjeldahl method with other products. A recently completed IDF study of the Kjeldahl method for protein in milk (3.2 g/100 g), using a copper sulfate catalyst, involving 24 laboratories in 12 countries and 12 test samples in a double-split-level design (6 assays), provides an estimate of RSD_R of 1.0 ± 0.1%, (1988) 0893PBJ. A similar 6-assay study of the Kjeldahl method for protein in cheese (3.5 g/100 g), using a copper catalyst (16) and involving 9 laboratories, shows an RSD_R of 1.8 ± 0.6%. The dye-binding methods for protein in various dairy products (about 3 g protein/100 g) in 4 studies show RSD_R values of 0.43–2.7% (omitting a value of 4.3% for cream and fat mixes), with the 2 average RSD_R values for milk assays being 2.7% (7 assays) and 1.4% (14 assays). Note the interesting similarity of the RSD_R values for protein to those of the Babcock method for fat; in both cases dissimilar analytes are measured by grossly dissimilar methods but at approximately the same concentration levels.

Boyer et al. (20), on the basis of studies of elemental analyses over 6 orders of magnitude of concentration, suggested that the empirical boundaries of independent estimates of RSD_R extend from 0.5 to 2 times the mean estimated value of RSD_R, values that differ by a factor of 4. This RSD_R estimate is probably as good a value as is available at the present time. Sufficient interlaboratory data may be available for the Kjeldahl method to provide a reasonable estimate of the distribution of the RSD_R values from collaborative studies of this method in numerous commodities, e.g., in fertilizers, feeds, cereals, and milk products (16, 17, 21) at comparable levels.

Throughout this series of examinations of the precision parameters of various methods of analysis applied to diverse sets of commodities (e.g., 10, 20), we have suggested that precision is a function of concentration only, independent of analyte, matrix, and method. This point can be demonstrated very convincingly by plotting the RSD_R value as a function of $-\log C$, as with the Horwitz curve, for the determination of fat (designated F) by all methods (gravimetric, volumetric, infrared, and automated) and for protein (designated by P) by all methods (volumetric, infrared, colorimetric) and drawing the convex hull for each analyte as in Figure 9. The convex hull is the envelope created by all the straight lines connecting every point in a set. Alternatively, the convex hull is the smallest polygon connecting all the extreme points in a set utilizing only convex angles (internal angles less than 180°). The only restriction that has been applied is not to include the extreme fat contents of skim milk (fat less than 2.5%), because the methods have not had sufficient collaborative testing at this level, and butter (fat about 80%), because there has been no comparable study of high milk protein products. In the present series of more than 200 assays for each analyte, the convex hulls (Figure 9) are remarkably similar. The moisture/solids data would also fit in this convex hull. They have not been included to avoid making the plot too crowded to read.

Combining a number of independent studies introduces the problem of the nature of the populations examined in the various studies. Does each study really contain a random sample of the laboratories that will be conducting analyses in the future with the method being studied? Or is each merely composed of a conglomeration of haphazard volunteers, good, bad, or indifferent, which yields a heterogeneous sample whose characterizing parameters depend on the mix of [analyst] populations? Since this mix of laboratories is not under statistical control, the combined ("pooled") results cannot be applied to any specific analyst or laboratory group when the derived parameters r and R are used. As indicated

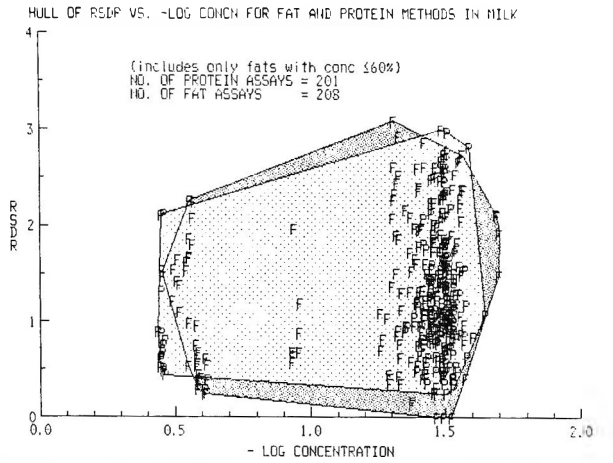

Figure 9. Convex hull of RSD_R as a function of $-\log_{10}C$ from fat and protein assays for milk products with fat and protein contents of about 2.5–35%.

by Hamaker (22), theoretically each assay should use a new, randomly drawn set of laboratories, but this he immediately points out is impractical, and the choice of laboratories should be "reasonably representative." Therefore, it can be stated only that the spread of results as measured, e.g., as percentiles, could be overestimating the precision parameters (less precise) according to a worst-case scenario of mixed populations. The conservative precision treatment suggested here presents ceilings for the precision parameters, i.e., the spread for any single laboratory or any 2 laboratories will not be any worse than that derived for the combined situation.

In circumstances where the laboratory population is under control, as with a group of control laboratories geographically scattered but under a single management, or a group of laboratories certified by a professional or regulatory body, and where continuous quality assessment is maintained, routine proficiency studies can develop specific parameters for the population of interest and ensure that they are kept in statistical control. Conversely, precision parameters imposed as part of an organizational, regulatory, or professional quality assurance program cannot be extrapolated to a population of laboratories not under the same system of quality assurance. Failure to take this distinction into consideration results in criticism of the collaborative study procedure for permitting lax criteria for laboratory performance on the one hand (23) and accusations of lack of realism in the field of analytical chemistry on the other (24).

The routine collaborative studies conducted by standards-making organizations such as the AOAC, however, for practical reasons, must take volunteer and invited laboratories as they come and must attempt to develop generalizations for acceptable precisions of the methods (and their applications by similar laboratories) that will keep the results in some semblance of statistical control. This series of papers (e.g., 10, 20) attempts to provide the basis for such generalizations. Some control of the collaborative study populations can be obtained in actual operation by the use of practice test samples that must be analyzed correctly before a laboratory (analyst) is accepted into the study. Better still would be a provision for the inclusion of benchmarks, systems suitability tests, reference constants, standard reference materials, and consistency checks as an intrinsic part of the written discriptions of analytical methods (25).

Conclusion

When a number of independent studies containing numerous assays for a constituent are available for developing

precision parameters, a characterless, unsymmetrical distribution of values is often obtained. Such distributions can be described in terms of the nonparametric precision parameters of the median and 90% interval of the relative standard deviation between laboratories, RSD_R. For the primary analytes (fat, moisture/solids, and protein) with concentrations of 2–100 g/100 g, the estimates of RSD_R center about 1%, occasionally dipping below 0.5% and rising above 4%. The 90% interval is about 0.3–3% and is independent of analyte, method, and matrix. Many methods of analysis for constituents of milk products have been accepted on the basis of single studies and even single assays. Some of these decisions may not be supported by the Referee but may be justified when the precision parameters appear to fall in the general range of acceptability.

In view of the range of RSD_R values for any given analyte-method-matrix combination, a specific point value for a precision parameter cannot be assigned. For analytes in the 1–100 g/100 g concentration range, as are all the major constituents and some minor constituents considered in this study, methods that show a pattern of RSD_R values well within a maximum of 4% can be considered as acceptable. Below this concentration and especially with instrumental methods, the Boyer criterion of a maximum of twice the RSD_R value calculated from the Horwitz formula of RSD_R (%) = $2 \exp(1 - 0.5 \log C)$, where C is expressed as a decimal fraction, may be considered as the guideline for the limit of acceptability of methods of analysis. Meeting these guidelines may be considered necessary but not sufficient for acceptability of a candidate analytical method.

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MYCOTOXINS

Use of Deuterated Internal Standards for Quantitation of T-2 and HT-2 Toxins in Human Blood by Tandem Mass Spectrometry

ROBERT J. PAWLOSKY, CHESTER J. MIROCHA, Y. WEN, and HAMED K. ABBAS
University of Minnesota, Department of Plant Pathology, St. Paul, MN 55108

Deuterated acetyl derivatives (3-trideutero-acetyl-T-2 and 15-trideutero-HT-2) were prepared for use as internal standards for the quantitation of T-2 and HT-2 in blood by tandem mass spectrometry. The method used was multiple reaction monitoring (MRM), which essentially involves the selection of a parent ion for analysis followed by monitoring of the daughter ions generated by collision activated decomposition. The parent ions chosen for the trifluoroacetate derivative of T-2 and HT-2 were m/z+ 478 and 532, respectively. Both parents yield the same daughter ions, i.e., 180, 138, and 121. HT-2 and T-2 were added to blood extracts in amounts ranging from 1 to 20 ppb. The limit of detection is about 0.5 ppb with an effective detection limit of 1.0 ppb in a range of 1–20 ppb. The recovery is about 90%. This method can be used by veterinarians for purposes of diagnostics. It can be used for urine as well as blood.

Trichothecenes are a group of secondary metabolites produced by various species of *Fusarium* and *Trichothecium*. The latter produce types A and B trichothecenes whereas *Myrothecium* produces the macrocyclic trichothecenes. Toxic effects in humans and animals caused by trichothecene-contaminated food or foodstuffs have been described in a number of reviews (1, 2).

T-2 toxin produced by the fungi *F. sporotrichioides*, *F. graminearum*, and *F. tricinctum* occurs naturally in cereal grains along with HT-2 toxin (Figure 1). In mammals, T-2 is metabolized to sesquiterpenoid compounds of lesser biological activity, such as HT-2, T-2 triol, and T-2 tetraol. The structures of these 4 compounds are identical except for deacetylation at C-3, C-4, and C-15 (Figure 1). The enzymes (deacetylation) present in heparinized whole blood are sufficiently active to change T-2 to HT-2 and T-2-tetraol even in refrigerated blood. Thus it usually is not sufficient when investigating cases of mycotoxicoses resulting from T-2 ingestion to analyze for T-2 alone. Analysis for the deacetylated products as well as T-2 is preferred.

Combined gas chromatography with tandem mass spectrometry (GC-MS-MS) is an instrumental system designed to utilize the resolution of capillary column chromatography combined with a multiple stage mass spectrometer to achieve a high degree of specificity and sensitivity. By properly selecting certain parents with high-yielding ion fragmentations (daughters), one is able to obtain higher signal-to-noise ratios than those of single stage selected ion monitoring. The term given to this kind of analysis is multiple reaction monitoring (MRM), which is the subject of the present paper (3, 4).

We report here an analytical procedure for the GC-MS-MS analysis of HT-2 and T-2 toxin in human blood extracts by using a deuterated internal standard of T-2 and HT-2 for quantification. The method is sensitive to as little as 1 ppb for the toxins in 5 mL blood extracts. Standard curves were generated for both toxins in the range of 1–20 ppb. The results of HT-2 analysis by using an internal standard were compared with results for an external nondeuterated standard procedure.

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Experimental

Sample Preparation

Five mL samples of whole human blood were amended with T-2 and HT-2 toxin at concentrations of 1, 10, and 20 ppb as well as 1, 10, and 20 ppb of the deuterated standards. Reverse-phase C-18 Bond-Elute minicolumns (500 mg) were used in the procedure. Columns were activated by rinsing with methanol (2 mL) followed by 2 mL water. Heparinized blood samples were precipitated with acetone (3 volumes of acetone to 1 volume of blood), and then were centrifuged 5 min at 7000 rpm. The blood was reextracted with acetone and the procedure was repeated. The acetone extracts were combined and concentrated on a steam bath. The extract was loaded onto a column and washed with 1 mL water. Toxins were eluted from the column with 2 mL acetone into ½ dram vials and concentrated under nitrogen at room temperature.

Five mL samples of monkey urine (obtained from monkeys dosed with T-2 toxin) were used for analysis. Bond-Elute (500 mg) columns were prepared as described above. Urine was loaded onto the column, washed with 1 mL water, and eluted with 2 mL acetone. The eluted material was concentrated to almost dryness in ½ dram vials and TFA derivatives were prepared as described below.

Deuterated Standard Preparation

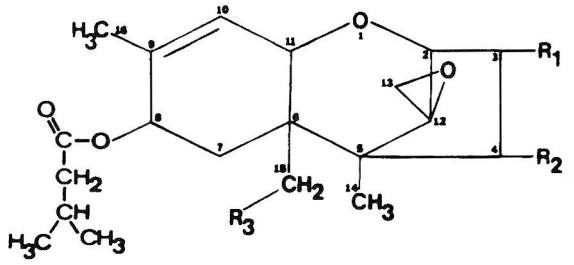
Deuterated T-2 and HT-2 toxins were synthesized in this laboratory by the method of Wen, Mirocha, and Pawlosky (paper in preparation). Essentially, T-2 toxin was acetylated with trideuteroacetic anhydride and selectively deacetylated to yield 4-deuteroacetyl-T-2. Deuterated HT-2 was made by synthesizing T-2-triol, acetylating with deuterated acetic anhydride, and then selectively deacetylating to yield 15-trideuteroacetyl HT-2 toxin. T-2 toxin was a gift from Robert Wannemacher (USAMRIID, Fort Detrick, MD) and Albert Pohland (Food and Drug Administration, Washington, DC). HT-2 was synthesized from T-2 toxin.

Derivatization

Reagent grade trifluoroacetic acid anhydride (Pierce Chemical Co., Rockford, IL) was used to derivatize the samples. Extracts were placed in ½ dram vials and 100 μL of reagent was added. Vials were capped and heated to 60°C for 20 min. Unreacted reagent was evaporated with a gentle stream of nitrogen. Twenty μL toluene was added to dissolve residue, and 1 μL solution was taken for injection into GC/MS/MS system.

Gas Chromatographic Conditions

Toxins were analyzed on a Hewlett-Packard 5890 gas chromatograph interfaced to a VG 7070EQ mass spectrometer. Samples were eluted from a narrow bore (0.25 mm), 10 m DB-5 covalently bonded phase capillary column, which was inserted directly into the source of the mass spectrometer. Injection port temperature was set at 250°C. The GC column was heated from an initial temperature of 80°C to



	R1	R2	R3
T-2 toxin	OH	OCOCH3	OCOCH3
Deutero-T-2	OH	OCOCD3	OCOCH3
HT-2 toxin	OH	OH	OCOCH3
Deutero-HT-2	OH	OH	OCOCD3

Figure 1. Chemical structures of trichothecenes T-2 and HT-2, as well as their deuterated derivatives.

280°C at 25°/min. Helium column flow measured prior to the column was 65 mL/min; at the source it was estimated to be 1–2 mL/min. Samples were introduced into the column by splitless injection with a 0.5 min delay at 80°C.

Mass Spectrometer Conditions

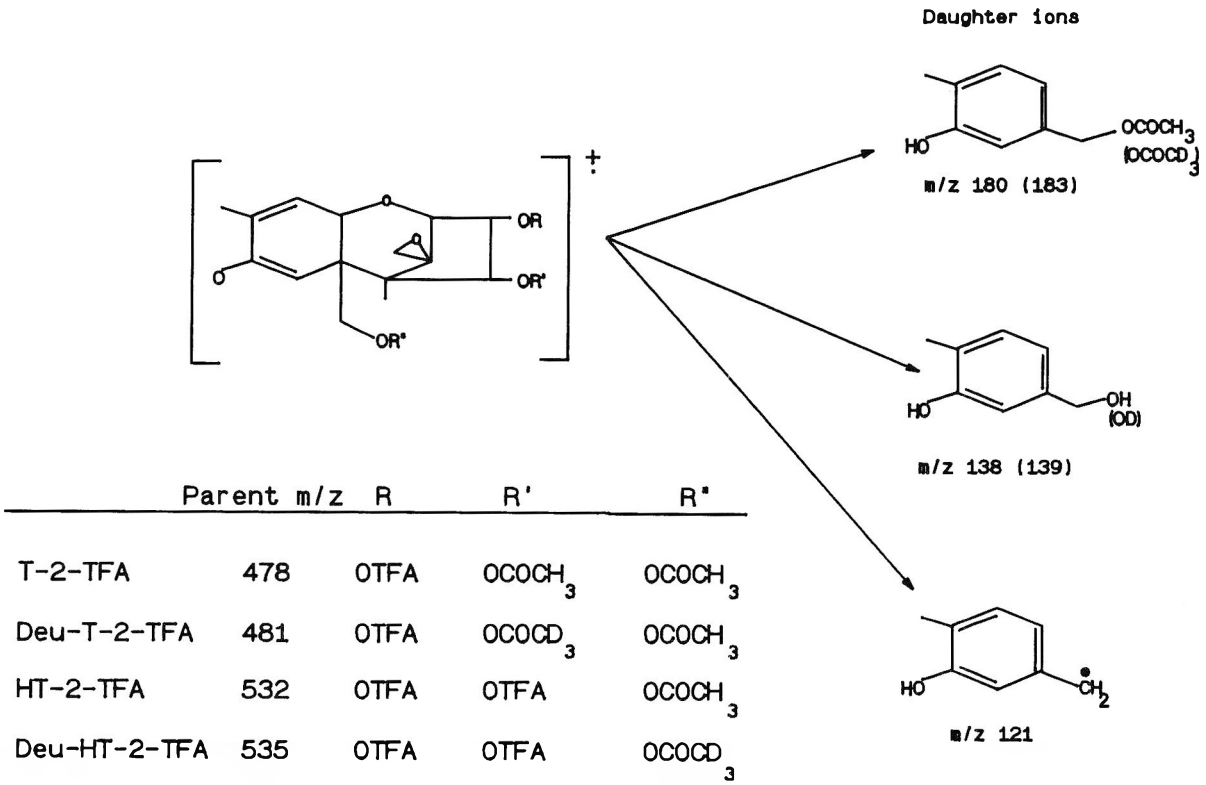
The ion source temperature was set at 125°C. All samples were run in 70 eV electron impact mode. Parent ions (478, T-2; 481, deuterated-T-2; 532, HT-2; 535, deuterated-HT-2) were selected by adjusting the accelerating voltage to allow passage of the specific ion through the electrostatic analyzer and the magnet. Instrument was programmed for 100 ms dwell time of each parent with a 10 ms switch time. Fragment ions (daughters) 180, 138, and 121 were used to detect both T-2 and HT-2. Deuterated T-2 and HT-2 standards were detected by using daughters 183, 138, and 121. Daughters of the deuterated standards and the toxins were identical except for m/z 183; however, the quantitative data for standards and toxins were kept separate, i.e., generated in different channels, so that quantitation was not confounded. The terminal quadrupole mass spectrometer was programmed to detect the different daughters in each channel selected. A dwell time of 100 ms was selected for each daughter with a 10 ms switching time.

The collision chamber (quadrupole mass spectrometer No. 1) was charged with argon (purity 99.999%) to maintain a pressure of 1×10^{-6} torr, read at a site remote from the collision chamber. The collision energy selected was 15 V.

Results and Discussion

The successful implementation of multiple reaction monitoring for T-2 and HT-2 analyses centers on the selection of appropriate parent/daughter combinations. Therefore, parent/daughter ion experiments were conducted to determine unique and sensitive combinations. To this end, parents at m/z 460, 400, 401, 478, and 327 were tested; parent 478 (although of low abundance) was chosen because it gave the greatest sensitivity, i.e., abundant daughter ions, and was the most diagnostic (4). Thus, after T-2-TFA undergoes the initial loss of a portion of the isovaleroxy group (84 amu) at C-8, to yield parent ion 478, the latter fragments to yield daughters 180, 138, and 121 in CAD. In a similar manner, HT-2-TFA loses a mass of 84 to yield m/z 532 (molecular ion 616 minus 84 = 532). The parent fragment 532 yields daughters 121, 138, and 180 after CAD (Figure 2).

The deuterated standards of T-2-TFA and HT-2-TFA both yield 121 as a daughter fragment (Figure 3) although they differ in that Deu-T-2-TFA yields 138 and 180 as daughters and Deu-HT-2-TFA yields 139 and 183 (Figure 4). The daughter m/z 139 is generated from C-15-trideuteroacetyl-HT-2 toxin after cleavage of the acetyl group between the ester oxygen and the carbonyl. There is a shift of



Parent	m/z	R	R'	R"
T-2-TFA	478	OTFA	$OCOCH_3$	$OCOCH_3$
Deu-T-2-TFA	481	OTFA	$OCOCD_3$	$OCOCH_3$
HT-2-TFA	532	OTFA	OTFA	$OCOCH_3$
Deu-HT-2-TFA	535	OTFA	OTFA	$OCOCD_3$

Figure 2. Fragmentation pattern of parents m/z 478 and 532 of T-2-TFA and HT-2-TFA, respectively, as well as their deuterated derivatives. Note that deacetylation of trideuteroacetate on C-15 results with hydrogen shift to remaining hydroxyl (m/z 139).

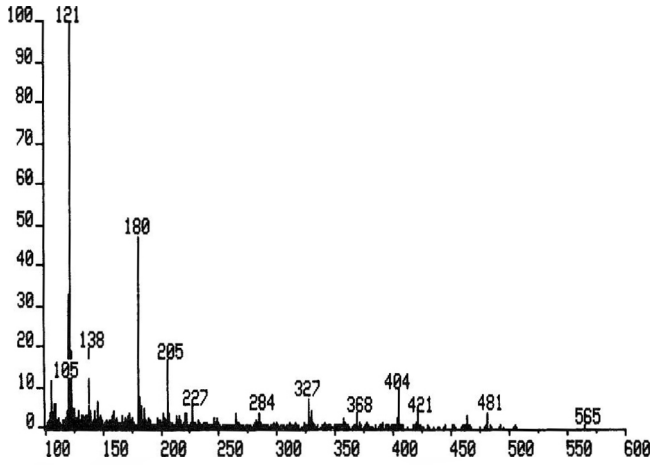


Figure 3. Electron impact full mass spectrum at 70 eV of C-4-trideuteroacetyl T-2-TFA. Molecular ion is 565.

one deuterium from the terminal methyl of the acetate to the remaining oxygen of the A ring. The advantage of the deuterated standards of both T-2 and HT-2 is that they undergo the same fragmentation as the principals (T-2 and HT-2), they coelute, and they have the same CAD conditions. However, quantitation of each fragment species, whether derived from the toxin or the deuterated standard, is kept in separate channels.

The advantage of multiple reaction monitoring (MRM) over selected ion recording (SIR) is that the analyst can select specific reactions, e.g., (parent 478 → 180, 138, 121) to resolve the analyte from the biological matrix. Moreover, SIR cannot discriminate the selected ions of the analytes vs the background matrix found in the biological samples. This can often lead to overlapping peaks that affect quantitation. More important, the use of only one ion in recording can lead to erroneous conclusions. In contrast, MRM is based on specific chemical reactions (occurring in the collision chamber), which result in the generation of signal peaks that are highly resolved and are more reliable than single stage detection.

Analysis of T-2 Toxin in Blood

T-2-TFA was resolved on a 10 m DB-5 capillary column. The full mass spectrum (electron impact) of deuterated T-2-TFA (Figure 3) shows m/z 481 and 404, confirming incorporation of the trideuteroacetate into the molecule. The total ion current (TIC) of the MRM reactions of human blood

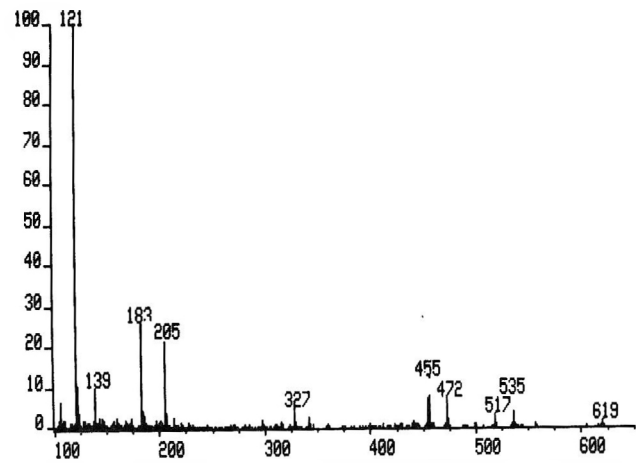


Figure 4. Full mass spectrum at 70 eV of C-15-trideuteroacetyl-HT-2-TFA. Molecular ion is 619.

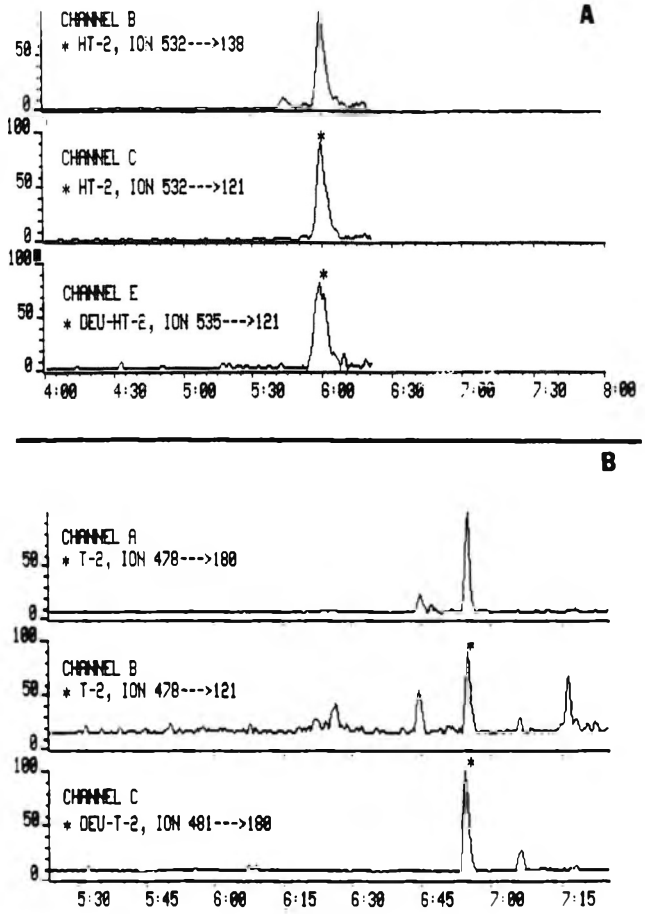


Figure 5. Total ion tracing of multiple reaction monitoring (MRM) of selected parent/daughter reactions.

(A) Channels B and C show parent 532 of HT-2-TFA giving rise to daughers 138 and 121 through collision activated decomposition. Channel E shows deuterated 535 giving rise to daughter 121 used in quantitation. (B) MRM experiment showing the total ion tracing of parent ion 478 giving rise to daughters 180 and 121 in channels A and B. Channel E shows deuterated parent m/z 535 giving rise to daughter 121 which was used in quantitation.

amended with 20 ppb T-2 toxin is shown in Figure 5B. Channels A and B show daughters 180 and 121, respectively, derived from parent 478 of T-2-TFA. The calculations were made from parent 481 of the deuterated T-2-TFA which generated daughter 180. Each chemical reaction is monitored in a separate data channel and, although identical fragments are obtained from different reactions, the results are processed separately.

Quantitation of T-2 toxin in human blood added at 1, 10, and 20 ppb was calculated on both external and internal standards as shown in Figure 6A and 6B. The accuracy and precision at 1 and 10 ppb for both the internal and external standards are excellent, whereas the accuracy at 20 ppb is acceptable but the precision has a standard deviation of 11.2. The actual data from which the figures were drawn are shown in Table 1. The average percent recoveries for 3 replicates, based on the internal standard at 1, 10, and 20 ppb, are 70, 72, and 80%. Both internal and external standards indicate greater precision at 1 and 10 ppb when compared with 20 ppb; the reason for this is not apparent.

Quantitation of HT-2 Toxin

The trifluoroacetyl derivatives of HT-2 and deuterated HT-2 were resolved on a capillary column and analyzed. The electron impact mass spectrum of the deuterated (15-trideuteroacetyl-HT-2) is shown in Figure 4. Mass fragments 183,

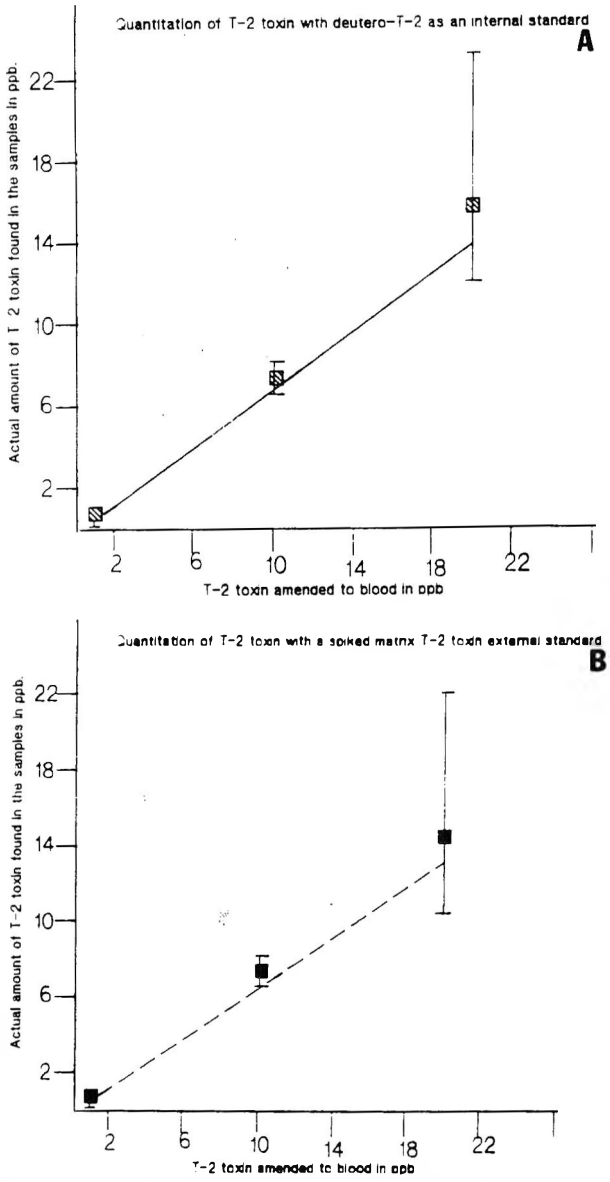


Figure 6. Analysis of T-2-TFA in human blood amended with T-2 at 1, 10, and 20 ppb: A, quantitation based on the deuterated internal standard; and B, quantitation based on external standard in biological matrix.

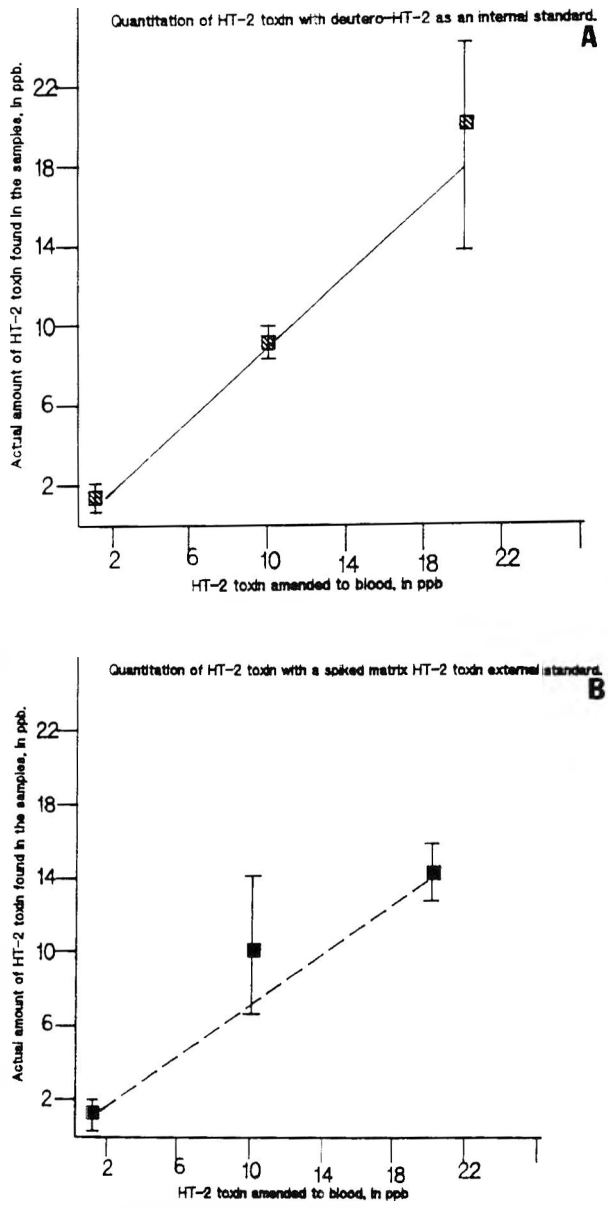


Figure 7. Analysis of HT-TFA in human blood amended with HT-2 at 1, 10, and 20 ppb: A, quantitation based on internal deuterated standard; and B, quantitation based on external standard in biological matrix.

Table 1. Quantitation of T-2-TFA toxin recovered from blood amended with T-2 to give final concentrations of 1, 10, and 20 ppb (ng/mL)[a]

	Area count					
Concn, ng/mL	T-2-TFA 478→180	Deut. T-2-TFA 481→180	Int. std, ng/mL	Rec., %	SD	Ext. Std, ng/mL
20	73	28	24	—		22
20	37	20	11	—		9
20	49	31	12	—		11
Av.			16	80	11.2	14
10	41	29	7.5	—		7.0
10	33	17	7.0	—		6.1
10	41	28	7.5	—		7.0
Av.			7.2	72	1.2	6.7
1	9	8	0.8	—		0.6
1	5	4	0.8	—		0.5
1	4	8	0.5	—		0.5
Av.			0.7	70	1.0	0.5

[a] Actual amounts injected into instrument were 4 ng/μL, 2 ng/μL, and 175 pg/μL. Each concentration consisted of 3 replications, i.e., each sample was spiked, extracted, and injected separately. Internal deuterated standard was used to calculate percent recovery.

Table 2. Quantitation of HT-2-TFA toxin recovered from blood amended with HT-2 to give final concentrations of 1, 10, and 20 ppb (ng/ml)[a]

	Area count					
Concn, ng/mL	T-2-TFA 478→180	Deut. HT-2-TFA 481→180	Int. std, ng/mL	Rec., %	SD	Ext. Std, ng/mL
20	135	206	13.1	—	—	14.0
20	149	146	20.1	—	—	15.1
20	187	211	25.2	—	—	12.5
Av.			19.3	97	4.7	13.8
10	76	66	8.9	—	—	14.0
10	85	76	8.9	—	—	10.1
10	104	109	9.2	—	—	7.0
Av.				90	0.17	10.4
1	45	80	0.6	—	—	2.0
1	32	17	2.1	—	—	1.5
1	60	72	0.8	—	—	0.5
Av.			1.4	140	0.96	1.3

[a] Each concentration consisted of 3 replicat ons. Calculations were based on an internal standard with deuterated acetate on the C-15 position.

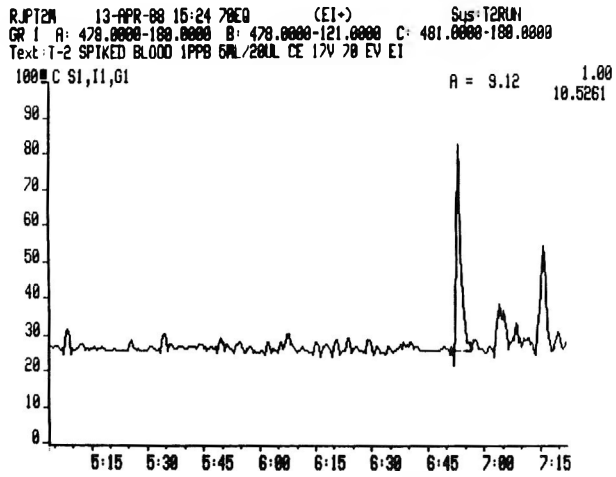


Figure 8. Total ion chromatogram of T-2-TFA in human blood amended with T-2 at 1 ppb. Reaction of parent 478 → 180 was monitored through MRM. Note excellent signal-to-noise ratio of 12:1 at retention time of T-2-TFA.

535, and 619 differ from HT-2-TFA by 3 mass units, thus confirming incorporation of the deuterated acetyl group. Channels B, C, and E (Figure 5A) show the total ion current derived from the reaction of parent ion 532 (HT-2) giving rise to daughters 138 and 121, whereas parent 535 (Deu-HT-2) generates ions 121, 139, and 183. The calculations were made on the integrated area counts of channel E (deuterated HT-2 535 → 121) and compared with channel C of HT-2 (HT-2 532 → 121). The plot of the quantitation using the deuterated internal standard is shown in Figure 7A. The accuracy and precision based on the internal standard at 1 and 10 ppb are excellent, whereas the precision at 20 ppb has a standard deviation of 4.7 (Table 2). The accuracy at 20 ppb, based on the internal standard, is superior to that of the external standard. The percent recoveries of blood amended with HT-2 at 1, 10, and 20 ppb are 116, 90, and 97%, respectively.

The limit of sensitivity of the method for the detection of T-2 toxin was calculated as being less than 1.0 ppb, based on the recovery of T-2 and HT-2 toxins from amended blood (Figure 8). The integrated averages of 1 ppb T-2 and HT-2 toxins in blood are 9 and 56, respectively; HT-2 is more easily detected. At a concentration of 1 ppb T-2 or HT-2 in blood and assuming 70% recovery, 175 pg is detected, although the limit of detection is less than 175 pg per injection. The peak height of the equivalent of 1 ppb T-2-amended blood is 49 mm with a signal-to-noise ratio of approximately 12:1, which illustrates the sensitivity of the method (Figure 8). The real advantage of MRM is the very low signal-to-noise ratio, which allows detection of very small amounts of analyte. Although MRM does not have any greater sensitivity than SIR, it is the high signal-to-noise ratio and elimination of chemical noise that allows detection of smaller absolute amounts. Signal-to-noise ratio becomes synonymous with sensitivity.

Analysis of T-2 and HT-2 Toxins in Urine

This method is also applicable to the analysis of T-2 and HT-2 from urine although it was not developed for that purpose. Table 3 shows the results of analyses of monkey urine for T-2 and its metabolites after the animal was dosed with T-2 toxin. Analyses were made both by selected ion recording (SIR) and multiple reaction monitoring (MRM) for comparison. The results in general are in good agreement,

Table 3. Analysis of monkey urine for T-2 toxin and its metabolites using deuterated T-2 as standard and multiple reaction monitoring, compared with analysis by selected ion recording[a]

	SIR, ppb		MRM, ppb	
Sample	HT-2	T-2	HT-2	T-2
1A56A	192	26	144	12
1A56B	157	226	285	132
1A56C	10	neg	5	neg
7C67A	65	87	35	30
81A67A	318	509	278	350
81A67B	16	12	42	25

[a] Analyses by SIR were made in chemical ionization (methane) mode on Hewlett-Packard 5987 quadrupole GC/MS. MRM determinations were made on VG 7070EQ equipment using method described in this paper.

with MRM showing values slightly lower than those of SIR. The latter does not discriminate selected ions from background as well as from the analyte and hence this factor might account for the difference.

Sample 1A56A in Table 3 shows the results of the analyses by SIR and MRM for T-2-TFA and HT-2-TFA. Analysis by SIR showed a value of 192 ppb for HT-2 and 26 ppb for T-2 compared to 144 ppb for HT-2 and 12 ppb for T-2 when analyzed by MRM. Although the values obtained are not identical, they are within the same order of magnitude. Sample 1A56C was negative for T-2 by both SIR and MRM, which indicates that false positives were not acquired. The samples analyzed by SIR were done on a Hewlett-Packard quadrupole instrument (HP5987B) in positive chemical ionization (in methane); those done on the VG 7070EQ instrument by MRM were done 14 months later. Although the samples analyzed were kept in a freezer and different aliquots were analyzed, it is surprising the close agreement in values that were obtained.

In the analysis of T-2 and HT-2 in the blood and urine of animals, the metabolites frequently encountered are HT-2, 3′-hydroxy-T2 (TC-1), 3′-hydroxy-HT-2 (TC-3), T-2-tetraol, and neosolaniol. The trifluoroacetyl derivatives of TC-1 and TC-3 yield doublet peaks (isomers) when resolved by capillary gas chromatography (5) but also yield the daughter ions 180, 138, and 121. TC-1 and T-2 contain m/z 478, and TC-3 and HT-2 contain m/z 532 as parent ions; all four yield the daughters 180, 138, and 121. These derivatives can be easily differentiated because their TFA derivatives can be resolved on a DB-5 capillary column. Although this analytical method was not intended for TC-1 and TC-3, they are easily detected and can be quantitated with the deuterated T-2 and HT-2 standards, respectively.

It is our opinion that multiple reaction monitoring using deuterated standards of T-2 and HT-2 is a unique and unequivocal method for the determination of T-2 and HT-2 in human blood and urine. This method is particularly accurate for measuring the low parts per billion range and can be adapted for use in both animal blood and urine studies. The method allows analyses of biological mixtures because it discriminates between interfering substances found in the matrix, resulting in a low signal-to-noise ratio. The method is also accurate in confirming the identity of T-2 and HT-2 because the parent fragments (478, 532) yield only daughter ions unique to T-2 and HT-2, respectively. Although other 478 and 532 fragments may be encountered in the analysis, they will yield different fragments in CAD and will not be detected.

Iso-T-2-TFA (3,15-diacetoxy-4-hydroxy-8-isovaleroxy-

12,13-epoxy-trichothec-9-ene) like T-2-TFA yields m/z 478 (daughters are found at m/z 121, 138, and 180) as one of its fragments; this metabolite can be distinguished from T-2-TFA by its retention time on a DB-5 capillary column but more definitively by the appearance of m/z 287 in the electron impact mass spectrum as opposed to m/z 227 for T-2-TFA. Thus the analytical method described is versatile enough to allow the detection of closely related derivatives of T-2 without misidentifying them.

The method herein described is versatile enough to be used by veterinary diagnostic laboratories in establishing probable cause of animal intoxication. It is reasonable to assume that analyses of body fluids of affected animals is more definitive than analysis of feeds for suspect mycotoxins. If the toxins are present in the blood or urine of the animal, it is clear that the animals must have ingested the toxin at least 24 to 72 h before the sample was collected.

Acknowledgments

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PESTICIDES AND INDUSTRIAL CHEMICAL RESIDUES

Isolation and Gas Chromatographic Determination of Chlorsulfuron in Milk

AUSTIN R. LONG, LILY C. HSIEH, MARSHA S. MALBROUGH, CHARLES R. SHORT, and STEVEN A. BARKER[1]

Louisiana State University, School of Veterinary Medicine, Department of Veterinary Physiology, Pharmacology and Toxicology, Baton Rouge, LA 70803

A method for the isolation and gas chromatographic determination of chlorsulfuron in milk is presented. Blank or chlorsulfuron-spiked milk samples were blended into C-18 (octadecylsilyl derivatized silica, ODS) packing material. A column made from the C-18/milk matrix was washed with hexane after which chlorsulfuron was eluted with dichloromethane (DCM). The DCM eluate contained chlorsulfuron which was free from interfering co-extractants when analyzed by gas chromatography utilizing a nitrogen/phosphorus detector. Chlorsulfuron was found to undergo a thermally induced decomposition to give 2-amino-4-methoxy-6-methyl-1,3,5-triazine, which was detected and quantitated by this method. Standard curves for these analyses were linear ($r = 0.992 \pm 0.004$, $n = 5$), with an average percentage recovery of $91.6 \pm 10.8\%$, over the concentration range examined (62.5–2000 ng/mL). The inter- and intra-assay variabilities were $11.6 \pm 7.5\%$ and 6.2%, respectively.

Chlorsulfuron [DPX4189, 2-chloro-*N*-[[4-methoxy-6-methyl-(1,3,5-triazin-2-yl)amino]carbonyl]benzenesulfonamide], is a selective pre- and post-emergence herbicide which belongs to the sulfonylurea class of compounds. As the active ingredient in Du Pont's Glean® herbicide, it finds application in wheat, oat, and barley production. Information regarding effectiveness of chlorsulfuron as a pre- and post-emergence herbicide as well as data related to its toxicology have been published (1). Even though the application rate is low (10–40 g/ha), the possibility of residues finding their way into food-producing animals such as cattle exists. Because chlorsulfuron may manifest itself in the meat or milk of grazing animals, the U.S. Department of Agriculture (USDA) Food Safety Inspection Service (FSIS) has included chlorsulfuron in the National Residue Program Plan (2). Monitoring of this residue may be required to ensure that tolerance levels [300 ng/g (2)] are not exceeded due to its inadvertent introduction into animal-derived foods.

Sulfonylurea therapeutic drugs have been analyzed by liquid chromatography (LC) (3, 4) and gas chromatography (GC) in blood, serum, and plasma (5, 6). Chlorsulfuron has been determined by LC analysis with photoconductivity detection (7, 8) and by GC analysis with electron capture, flame ionization, and nitrogen/phosphorus detection (NPD) of derivatized samples (9). The isolation of chlorsulfuron from soil (7), plant material (8), and water (9) has required the use of large volumes of extracting solvents and pH adjustments to achieve recoveries from 65 to 87%.

Methods for the analysis of chlorsulfuron as a residue in a complex matrix such as foods have been less apparent in the literature. Because of the lack of sensitivity when photoconductivity or ultraviolet (UV) detection is used with LC analysis, and the occurrence of multiple derivatives for GC analysis (9), we investigated the possibility of analyzing the thermal decomposition products of underivatized chlorsulfuron by GC/NPD. Work in this laboratory (10) has shown that unique fragments are formed from the temperature-induced decomposition of chlorsulfuron and that these fragments can be quantitatively determined. Thus, we describe here a method based on matrix solid-phase dispersion [MSPD (11, 12)] extraction and GC/NPD detection and quantitation for the determination of chlorsulfuron as a residue in milk.

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[1] Address correspondence to this author.

Experimental

Reagents

(a) *Solvents.*—Obtained from commercial sources; highest purity available and used without further purification.

(b) *Chlorsulfuron.*—DPX4189; supplied by E. I. Du Pont de Nemours and Co. (Wilmington, DE).

(c) *Bulk C-18 column packing.*—Octadecylsilyl derivatized silica, 18% load, endcapped, 40 μm (obtained from Analytichem, International, Harbor City, CA). The C-18 was cleaned by sequentially washing 50 mL column, containing bulk C-18 (22 g), with 2 column volumes each of hexane, dichloromethane (DCM), and methanol by vacuum aspiration until dry.

(d) *Milk.*—3.2% butterfat, vitamin D, homogenized (obtained from local market).

(e) *Chlorsulfuron stock standard solutions.*—3.12, 6.25, 12.5, 25, 50, and 100 μg/mL. Prepared by dissolving pure standard in methanol and diluting with methanol to appropriate ng/mL level. Stock solutions were stored in amber bottles at −5°C to prevent decomposition. The ng/mL levels studied are given in Table 1.

Preparation of Sample Extracts

Two g C-18 were placed in a glass mortar and 0.5 mL milk was added. Chlorsulfuron (10 μL of a given stock standard solution) was added to the milk. Methanol (10 μL) containing no standard was added to the blank milk sample. Samples were allowed to equilibrate 1 min and then were blended into the C-18 packing with a gentle circular motion (30 s) using a glass pestle. Alternatively, milk sample was placed in the mortar, spiked, and allowed to equilibrate, and the C-18 was added and then blended. These 2 approaches gave equivalent results.

The homogeneous C-18/milk matrix was transferred to a 10 mL syringe barrel which had been plugged with a filter paper disc (Whatman No. 1). The column containing the C-18/milk matrix was covered with a filter paper disc (Whatman No. 1) and compressed to a final volume of 4.5 mL with a syringe plunger from which the rubber end and pointed plastic end had been removed. The column was washed with 2 column volumes (9 mL) of hexane by gravitational flow. When flow had ceased, excess hexane was removed by applying positive pressure (pipet bulb) to the column head.

Chlorsulfuron was eluted by passing 2 column volumes of dichloromethane (DCM) through the column as described above for hexane. The DCM extract was dried under a steady flow of dry nitrogen gas. The residue was solubilized with 250 μL DCM, and an aliquot (2 μL) was analyzed by gas chromatography. Operating conditions: Varian Vista 6000; DB-5 column (J & W Scientific), 25 m × 0.25 mm id, 0.2 μm

Table 1. Recoveries of chlorsulfuron isolated from spiked milk samples

Spike concn, ng/mL	Recovery, % (n = 5)
62.5	78.6 ± 18.8
125	101.9 ± 12.9
250	77.9 ± 12.7
500	101.1 ± 15.6
1000	97.1 ± 5.2
2000	93.1 ± 3.4
Corr. coeff., %	0.992 ± 0.004
Inter-assay var., %	11.6 ± 7.5
Intra-assay var., %	6.2
Recovery, %	91.6 ± 10.8

coating. Column temperature program: 90°C for 1 min, increase at 20°/min to 250°C, and hold for 1 min. Splitless injection with purge function activated at 0.75 min post-injection. Injection port 250°C. Detector: nitrogen/phosphorus, 300°C, 16 mV at 32×10^{-12} sensitivity.

Peak areas of standards and spiked sample extracts, run under identical conditions, were compared to determine percentage recoveries at each concentration examined. Intra-assay variability was determined by the standard error of the mean of 5 replicates of an identical sample. Inter-assay variability was determined as the mean of the standard error of the mean of five 6-point sample standard curve areas.

Results

Representative chromatograms of an extracted milk blank and chlorsulfuron-spiked milk sample are shown in Figures 1A and 1B, respectively. Table 1 gives the concentrations examined, correlation coefficient (± standard deviation), percent recovery, and inter- and intra-assay variabilities of chlorsulfuron isolated from spiked milk samples.

Discussion

Solid-phase extraction has been used to isolate chlorsulfuron from water samples (13) with excellent results. The isolation of chlorsulfuron from milk by using a modified SPE technique as outlined here, which we have named matrix solid-phase dispersion (MSPD), yields extracts which have a minimum of interfering co-extractants (Figure 1A). A GC chromatogram (Figure 1B) of a chlorsulfuron-spiked sample indicated that a detectable product of chlorsulfuron did not co-elute with other extractants found in the milk blank (Figure 1A). The product quantitated is 2-amino-4-methoxy-6-methyl-1,3,5-triazine (10), as characterized by GC-mass spectroscopy (GC-MS) and GC-Fourier transform infrared spectrometry [GC-FTIR (10)].

The presence of the triazine in samples prior to extraction does not interfere with the analysis. Triazine is not extracted from milk samples using the conditions described here but is only eluted under conditions using solvents of greater polarity than DCM, such as ethyl acetate (14). The second decomposition product, *o*-chlorobenzenesulfonamide, was not detected by GC-NPD but was confirmed by GC-MS and GC-FTIR (10).

The isolation of chlorsulfuron from the C-18/milk matrix, as outlined here, eliminates the need for large volumes of extracting solvents and/or pH adjustments, which is typical in classical isolation techniques. The approach is a further application of work done previously in this laboratory (11, 12) using a C-18/tissue blend for drug isolations. The sample is distributed over a large surface area, thereby exposing the entire sample to the extraction process and eliminating the classical homogenization of the sample in an extracting solvent. We theorize that the lipid materials in the milk associate with the lipophilic C-18 polymer and that proteins align in such a way as to allow lipophilic regions to associate with the lipid and/or lipid/C-18 regions, with the more polar ends extending outward. The polar hydrophilic protein regions then preferentially associate with the water and water-soluble components of the milk. The distribution is probably discontinuous in nature, allowing one to selectively elute different classes of compounds. Scanning and transmission electron micrographs of uncoated and tissue-coated C-18 beads support this hypothesis (unpublished observations).

Although derivatization of sulfonylurea type compounds, which includes chlorsulfuron, has been suggested as a prerequisite for gas chromatography, we found that the thermal

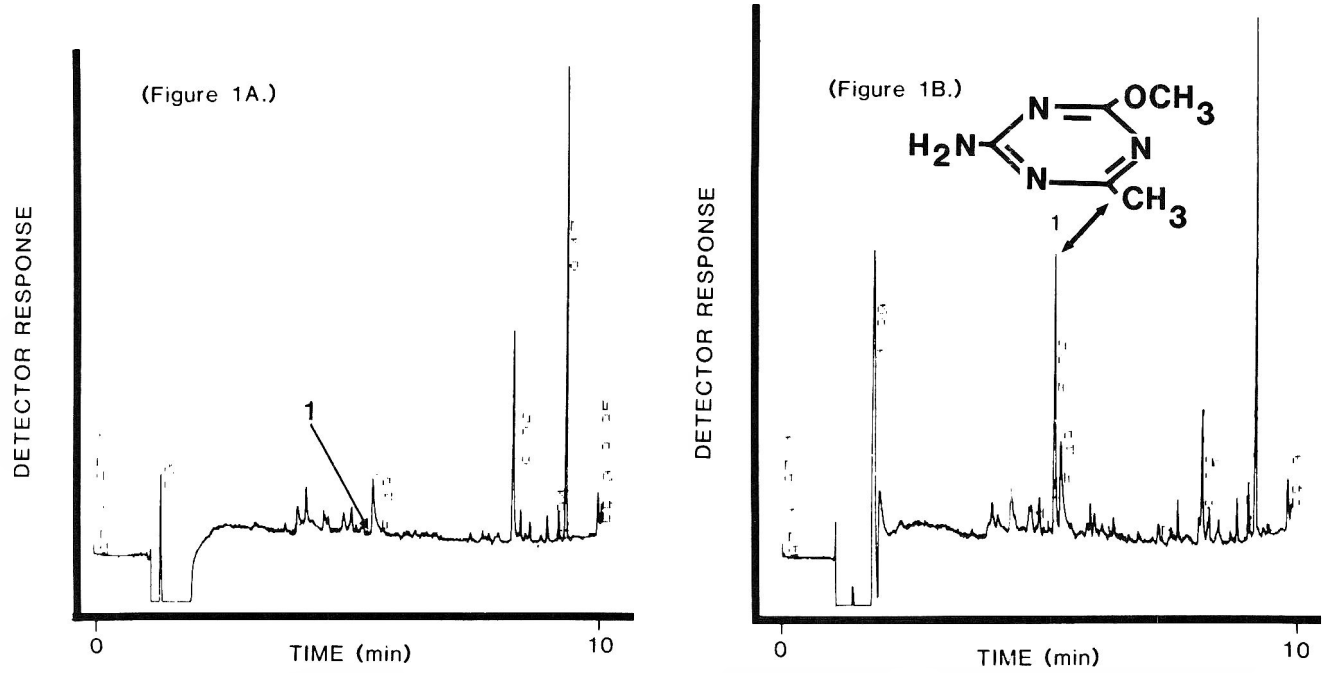

Figure 1. Representative chromatograms obtained from the GC-NPD analysis: A, dichloromethane extract of blank milk; and B, chlorsulfuron-spiked milk (peak number 1, 2-amino-4-methoxy-6-methyl-1,3,5-triazine) (500 pg on-column).

decomposition products of chlorsulfuron chromatographed well under the conditions outlined here. The technique eliminates the need for derivatizations and the inherent problems of derivative degradation and multiple derivative products. Utilization of nitrogen/phosphorus selective detection minimizes interferences that may be inherent to the sample extracts and provides a means by which chlorsulfuron can be quantitatively determined. Furthermore, identity may be confirmed by gas chromatography-mass spectrometry (10).

The isolation technique described here may be applicable to chlorsulfuron determinations in other matrixes. The technique requires small sample sizes (0.5 mL) and small volumes of extracting solvents which make this method attractive when compared to classical extraction techniques. Extract pH adjustments and backwashing are avoided but could be used to further clean up extracts obtained by this method, if needed. Using this method, we were able to quantitatively detect levels as low as 100 pg on-column (50 ng/mL), which is comparable to the methods of Zahnow (7) who used LC analysis with photoconductivity detection and Kelley (15) who reported the development of an immunoassay technique for chlorsulfuron. Detectable limits reported by other researchers (3-6, 8) were in the ng range. The use of internal standardization may also facilitate the quantitative determinations presented here. In addition, other sulfonylurea compounds may be isolated from plasma, serum, or blood samples by a similar approach and their pyrolytic products may be determined in a similar manner.

Acknowledgments

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Determination of Methyl Bromide in Foods by Headspace Capillary Gas Chromatography with Electron Capture Detection

B. DENIS PAGE and RICHARD J. AVON
Health and Welfare Canada, Health Protection Branch, Food Directorate, Ottawa, Ontario K1A 0L2, Canada

Methyl bromide (MB, bromomethane) is determined in a variety of foods by headspace capillary gas chromatography with electron capture detection. The comminuted food sample as an aqueous sodium sulfate slurry is equilibrated with stirring for 1 h at room temperature before a 1 mL headspace aliquot is removed and injected using a modified on-column syringe needle. Methyl bromide is cryogenically focussed at −60°C and then eluted by temperature programming. The procedure requires blending of soft samples, e.g. raisins, prunes, or oranges, and ultrasonic homogenization of hard samples, e.g. wheat, cocoa beans, corn, or nuts, with portions of water and ice so the final temperature of the food–water slurry is < 1°C. A 20 g aliquot (4 g food) is then added to a cold headspace vial containing 4 g sodium sulfate. Losses of MB during a 3.5 min ultrasonic homogenization of wheat were 11% at 0.95 ppb and 4.4% at 4.8 ppb. For flour, cocoa, and finely divided spices, which do not require blending, 4 g is added to the cold headspace vial containing 16 mL cold water and 4 g sodium sulfate. Studies show that comminution of wheat or peanuts must be carried out to release MB trapped within the food so the headspace equilibrium can be attained in 1 h as well as to obtain homogenous samples and representative sampling. No interferences were noted with the above foods or with many grain-based baking mixes analyzed. Detection limits were shown to be generally dependent on the approximate lipid content of the food and ranged from 0.15 to 0.65 ppb (5% full scale deflection). Repeatability CVs for spiked or incurred MB in flour, wheat, and parmesan cheese at 0.60–96 ppb were < 6.8%.

With the severe restriction in the use of the fumigant ethylene dibromide (EDB, 1,2-dibromethane) in 1984, public and regulatory attention has been directed to the use of other fumigants and insect control measures. It was expected that other permitted fumigants, such as methyl bromide (MB, bromomethane) would find increased use (1) and analytical procedures would be needed to monitor the expected residues in a wide variety of food products at low ppb levels.

The analysis of fumigants as a class of volatile compounds has been addressed by several approaches. These include distillation, cold solvent extraction or soaking, and headspace analysis. The determinative step in each of these procedures almost always involves gas chromatography (GC) with electron capture (EC) detection. Distillation has been applied to fumigants such as EDB, carbon tetrachloride, ethylene dichloride, chloroform, and carbon disulfide (2-4) but not to MB. This is presumably due to the volatility of MB (bp 3.6°) which makes MB difficult to contain in open distillation systems.

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A cold solvent extraction or soaking procedure has been developed by Heuser and Scudamore and shown to be applicable to MB at 0.3 ppm (5, 6). They determined the optimum extraction periods for MB from flour and unground wheat, sultanas, peanuts, maize, groundnuts, and cocoa beans to be 1, 8, 8, 24, 24, and 48 h, respectively. The extraction time increased with food particle size. With several foods, evidence of MB loss due to reaction with food components was noted.

Recently, Scudamore (7) reevaluated the cold solvent extraction procedure and determined that, for many fumigants, complete recovery from aged unground cereal grains required extended extraction times, well beyond those recommended earlier. This probably reflects the migration of the fumigant to the interior of the grain. Fairall and Scudamore (8) also improved the sensitivity of the cold solvent extraction procedure for MB by converting the MB in the extract to methyl iodide, which gives a much greater EC response. Thus, they were able to determine MB as a derivative at levels as low as 10 ppb. The disadvantages of the extraction procedure are the prolonged 24 h extraction period recommended for complete extraction of MB and, despite the derivatization step for enhanced sensitivity, the relatively dilute sample extract which results in unexpected poor sensitivity.

The headspace approach has been used to determine MB in selected foods but well studied procedures applicable to a wide variety of samples are lacking. King et al. (9) described a headspace procedure to determine MB in grapefruit at levels as low as 2 ppb. A 500 mL blender container with a screw lid fitted with a silicone septum was used as the headspace container. A 50 g portion of grapefruit was blended with 50 mL water for 1 min. After 20 min standing, a headspace aliquot was injected into the gas chromatograph via a 0.5 mL sample loop. Their procedure was also reported applicable to other fruits and vegetables, although pertinent data were lacking.

More recently, DeVries and co-workers (10) proposed a headspace procedure for MB in flour, cocoa, and unground wheat and peanuts. In their procedure, 1 g sample is equilibrated with 2 g water and the headspace for 2–12, 1–12, 1–10, and 6–17 h, respectively. These equilibration time windows are designated as the time period over which the headspace sample response remains somewhat constant before a substantial decrease occurs. The windows were determined by analysis of in-house fumigated samples of each commodity which had been left standing for various time intervals. Studies determining the equilibration time windows in reverse, i.e., aqueous MB to food and to headspace, however, gave 1–10 or 2–10 h periods for the same foods.

In our laboratory, it was necessary to develop a procedure suitable for the routine monitoring of low levels of MB in wheat, flour, other whole and ground cereals, cheese, fruit, and other food products which may possibly be fumigated with MB. The headspace technique appeared to offer the best potential for both good sensitivity and high sample throughput and was chosen as the procedure to be developed. Capillary gas chromatography with EC detection was expected to give the best sensitivity and selectivity for the determinative step. For sample preparation, it was decided to study blending of the sample with dry ice or water-ice. This blending would minimize most problems associated with determining suitable equilibration times for different foods as the leaching/equilibration time would be expected to be similar for any comminuted sample, even if the sample was aged. Similarly, the effect on repeatability of a wide variation of fumigant residue levels between individual grain kernels described by Daft (11), could be minimized. Furthermore, any time-dependent loss of MB by reaction with the food during headspace equilibration would be reduced with decreased contact time. Studies using actual fumigated samples with incurred MB were done to evaluate proper sample preparation prccedures and to establish headspace equilibration times so that repeatable maximum recoveries from a wide variety of samples could be obtained.

METHOD

Apparatus

(a) *Gas chromatograph.*—Varian Vista Model 6000, or equivalent equipped with electron capture detector, cryogenic oven cooling, on-column injector, and DB-1701 (1 μm film thickness) 15 m × 0.32 mm capillary column (J&W Scientific Inc., Folsom, CA 95630). Operating conditions: temperatures (°C) injector 60, detector 275, oven temperature program −60 to −20 at 40°/min, −20 to 0 at 5°/min, and 0 to 80 at 40°/min, with hold at 80°C for 3 min. Helium carrier gas at 2.3 mL/min (47 cm/s) and nitrogen make-up gas at 35 mL/min. Provided satisfactory performance is achieved, other equipment and conditions may be used such as: splitless injection, other capillary columns (≥ 1 μm film recommended), as well as other oven temperature programs and gas flow rates. Retention time for MB should be 2–3 min. An injection of 1 mL headspace from over an aqueous sodium sulfate solution containing 8 ng should give peak height of ca 50% at 16×.

(b) *Headspace sampling syringe.*—1 mL gas-tight locking syringe series A-2 (Precision Sampling Corp.) as shown in Figure 1A, or equivalent, fitted with 2 in. sideport needle (Figure 1B).

(c) *On-Column injection needle assembly.*—As shown in Figure 1C. Cover butt end of Varian sheathed on-column injection needle with 1 cm piece of 1.5 mm od × 0.3 mm id Teflon tubing (Pierce Chemical Co.). Cut sideport syringe needle from (b) above to ca 2 cm and cover with 2 cm piece of above tubing. Connect on-column injection needle to sideport needle hub with 1/16 in. stainless steel Swagelok union using Teflon ferrules. Other equivalent gas-tight syringe and needle assemblies may be used.

(d) *Gas-tight syringe.*—100 μL model 1800 (Hamilton and Co.), or equivalent.

(e) *Headspace sample vials.*—30 mL Hypovials (Pierce Chemical Co.), actual capacity ca 37 mL, or equivalent, sealed with Teflon-laminated silicon rubber discs (Pierce Chemical Co.), or equivalent. Discard disc after single use. Crimp headspace sample vials tightly so aluminum cap cannot turn readily.

(f) *Magnetic stirring bars.*—Teflon-coated, 25 × 7.5 mm for (e) above.

(g) *Sampling tube for dry foods.*—Fit 20 cm × 9 mm id glass tube with 22 cm × 9 mm od glass rod. Fit 1 cm piece of Tygon tubing over end of rod.

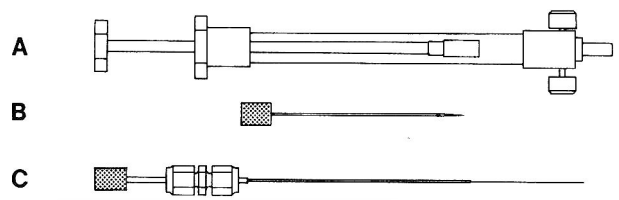

Figure 1. A, 1 mL gas-tight syringe with B, 2 in. sideport needle for headspace sampling, and C, modified needle assembly for on-column capillary injection.

(h) *Blender.*—Waring or equivalent, with 1.0 or 1.2 L container for homogenizing soft samples.

(i) *Homogenizer.*—Polytron Model PCU-1, or equivalent, equipped with PTA-20 single-hole anaerobic probe generator for homogenizing hard grain samples. Remove top of T 27 hollow plastic stopper and cut ca 2 cm diameter hole in stopper body to snugly fit probe.

(j) *Polyethylene bags.*—5 × 3 × 14 in. or similar size.

(k) *Slurry sampler.*—Cut stopcock off 25 mL glass buret. For low viscosity food slurries, fit wide end of sampler with suitable tubing adapter so propipet can be used, and dispense sample through constricted end; for more viscous slurries, fit propipet to narrow end of sampler and dispense sample through wide end.

Reagents

Caution: Methyl bromide is toxic. Observe necessary safety precautions. Carry out all manipulations except sample spiking in fume hood.

(a) *Methyl bromide (MB).*—Bromomethane (99.5% in lecture bottle from Matheson Gas Products, Canada). Connect control valve to hypodermic syringe fitted with 23 gauge 1½ in. needle using short length of Tygon tubing.

(b) *Water.*—Using electric kettle, boil distilled water 15 min to remove interferences. An injection of 1 mL equilibrated headspace from over 20 mL distilled water and 4.0 g sodium sulfate should not show any peaks interfering with MB.

(c) *Ice cubes.*—Prepare from boiled water.

(d) *Acetone.*—Distilled-in-glass grade.

(e) *Sodium Sulfate.*—Anhydrous, granular, reagent grade.

(f) *Mercury.*—Reagent grade.

(g) *MB stock solution.*—1–2 mg/mL. Pipet 30 mL acetone into headspace vial containing 10 g mercury. Seal with septum and aluminum seal. Record weight to nearest 0.01 mg. Purge air from tubing attached to MB lecture bottle, open lecture bottle control valves, and purge air and MB into 25 mL Erlenmeyer filled with methanol until air bubbles are no longer seen. Insert a needle, e.g. 23 gauge 1½ in., just through septum as vent. Insert MB needle through septum into acetone and allow MB gas to flow into acetone for 30–60 s. Remove needles and turn off valves. Invert headspace vial and weigh to nearest 0.01 mg. Determine weight and concentration of MB. If less than 1 mg/mL, add more MB as before. If greater than 3 mg/mL, repeat preparation of stock solution using lower flow rate of MB. Always keep headspace vial containing stock solution inverted so mercury covers septum.

(h) *MB spiking solutions.*—Prepare fresh daily. Keep all flasks immersed in ice-water bath. Use water aspirator vacuum to fill pipets. Use chilled boiled distilled water for all dilutions. (*1*) 20 μg/mL. Using 100 μl syringe, sample and inject 100 μl MB stock solution (g) into 10 mL volumetric flask containing ≤ 9.9 mL water. Dilute to volume and mix. (*2*) 200 ng/mL. Pipet 1 mL of solution (*1*) into 100 mL volumetric flask containing ≤ 99 mL water. Dilute to volume and mix. (*3*) 100 and 40 ng/mL. Pipet 5 or 2 mL of solution (*2*) into separate 10 mL volumetric flasks containing ≤ 5 and ≤ 8 mL water, respectively. Dilute to volume and mix. (*4*) 20, 8, 4, and 2 ng/mL. Pipet 1 mL of solution (*2*) into each of 10, 25, 50, and 100 mL volumetric flasks containing ≤ 9, 24, 49, and 99 mL water, respectively. Dilute to volume and mix.

Sampling of Homogeneous Foods

When received, store packaged dry foods such as cocoa, flour, ground spices, and other finely divided foods which are homogeneous in freezer at −20°C. Add to each headspace via 17 mL distilled water, 4.0 g sodium sulfate, and a magnetic stirring bar, and cool in water-ice bath at least 15 min. Tare headspace vial on top-loading balance, open sample package, and repeatedly plunge glass sampling tube with plunger retracted into the food to fill tube. Wipe off outside of tube with disposable tissue, insert end of sampling tube into headspace vial on top loading balance, and depress plunger to dispense ca 4 g sample. Repeat sampling for duplicate samples. Determine weight of samples to 0.01 g and immediately seal each vial with septum and aluminum seal. Place sealed headspace vial in 150 mL beaker containing ca 60 mL water at ca 45°C and stir. Remove beaker after 15 min and continue stirring. After 1 h at room temperature, sample headspace as described below in *Headspace Technique.*

Sampling of Nonhomogeneous Foods

Store nonhomogeneous dry foods or foods of large particle size such as dates, nuts, or prunes in freezer at −20°C overnight; store oranges or other fruit at 4°C in refrigerator.

(a) *Small particle size samples (e.g., corn, wheat, cocoa beans).*—Crush ice cubes inside double polyethylene bag, place 250 mL T 27 Erlenmeyer flask on top-loading balance, and add ca 85 g crushed ice and then room temperature boiled distilled water to make 160.0 g. Mix food sample in package by shaking, open cold sample package, mix with spatula, transfer 40 g peanuts, wheat, corn, or similar or small particle size sample to container on balance, and determine weight to nearest 0.1 g. Insert homogenizer probe to within 0.5 cm of Erlenmeyer bottom and adjust stopper to fit neck. Homogenize sample until homogeneous slurry is obtained. Adjust water-ice ratio and repeat sampling and blending if necessary so that ice is just melted when blending is completed and sample temperature is ≤ 1°C. Working quickly, chill slurry sampler by filling with sample. Discard sample, refill sampler, and immediately transfer 20 g sample in duplicate to tared headspace vials (top-loading balance) containing 4.0 g sodium sulfate, 1 mL distilled water, and magnetic stirring bar, and proceed as in *Sampling of Homogeneous Foods*, starting "Determine weight of sample to nearest 0.01 g . . .". Viscous slurries, e.g. with cocoa beans or mustard seed, require dilution so that effective stirring in headspace vial can be carried out. With these samples, pipet 10 mL water into headspace vial and chill before adding 10 g sample slurry.

(b) *Large particle size samples (e.g. prunes, dates, citrus fruits).*—Cut prunes or dates into small pieces, and cut citrus fruits into representative quarter or smaller segments; add ca 100 g prunes or dates or 150–200 g citrus fruit segments to tared blender on top loading balance. Determine weight to 0.1 g. Crush ice cubes in double polyethylene bag and immediately add about 1.5 times sample weight. Add room temperature boiled distilled water to give ice + water weight exactly 4 times sample weight. Blend until homogenous slurry is obtained Continue as in (a) above, starting "Adjust ice-water ratio and repeat . . .".

Preparation of Calibration Curves

Verify absence of MB in each food by conducting blank determinations for homogeneous or nonhomogeneous foods as described below. To each of 11 headspace vials, add 4.0 g sodium sulfate and magnetic stirring bar. For homogeneous foods, add 16 mL distilled water and 4.0 g food not containing methyl bromide to each of the 11 vials. For nonhomogeneous foods, add to each of the 11 vials 20.0 g slurry prepared

as in (**b**) above, using food not containing methyl bromide. Cool in water–ice bath for at least 15 min. For MB levels in foods to 10 ppb, add duplicate 1 mL aliquots of MB spiking solutions 40, 20, 8, 4, and 2 ng/mL; for MB levels to 50 ppb, use 200, 100, 40, 20, and 8 ng/mL MB solutions to give concentrations of 10, 5, 2, 1, and 0.5 or 50, 25, 10, 5, and 2 ppb, respectively, with respect to the food. Add 1 mL distilled water to 1 vial as blank. Immediately seal each vial with septum and aluminum seal after each spike. Place sealed headspace vial in 150 mL beaker containing ca 60 mL of water at ca 45° and stir. Remove beaker after 15 min and continue stirring. After 1 h at room temperature, sample headspace as described below in *Headspace Technique*.

Using chromatogram of blank sample as guide, draw baseline and estimate peak height of methyl bromide (H_{MB}) to nearest 0.1 mm or determine peak area (A_{MB}) by electronic integration. Correct for any interfering peak in blank sample. Plot average H_{MB} or A_{MB} vs ppb MB and draw curve through origin and points.

Headspace Technique

Refer to Figure 1. Flush barrel of syringe (A) and sideport needle (B) with nitrogen. Replace plunger and pierce septum of sealed headspace vial inserting needle about 1–2 cm. Withdraw plunger slowly (5 s) to beyond 1 mL mark. Wait 10 s for barrel to fill. Align plunger tip with 1 mL mark. Lock syringe and withdraw needle. Remove sideport needle (B) and replace with on-column injection needle assembly (C) for headspace injection. Insert fused silica needle into on-column injector, unlock syringe, and inject sample onto column. Start temperature program. Identify MB by retention time.

Calculation

Measure peak heights (H_{MB}) or determine areas (A_{MB}) of MB for duplicate samples as for calibration curves. Determine ppb MB from calibration curve and correct to 4.00 g sample.

Results and Discussion

In developing a procedure to determine MB in foods, it was first necessary to determine the chromatographic conditions so that MB eluted in a reasonable time with acceptable peak shape. It was recognized that MB in a 1 mL headspace sample would need a reduced column temperature as well as a capillary column with a relatively thick film so that MB could be concentrated or focussed as a narrow band on the head of the column; MB could then be eluted as a sharp peak with a temperature program. Initial trials were conducted using a column with a 1 μm film, a flow rate of 2 mL/min (47 cm/s), and on-column injection. The on-column injector required that the Varian fused-silica sheathed needle be adapted to fit a standard gas-tight syringe as shown in Figure 1. It was determined that an oven temperature of at least −50°C was necessary to concentrate (focus) the MB contained in the 1 mL headspace so that band broadening did not occur as a result of the injection. With low μL headspace injections, peak tailing or broadening did not occur. The oven temperature was then rapidly (50°C/min) raised to −20°C, and MB eluted during a slow (5°C/min) temperature program.

Figure 2 shows the effect of initial oven temperature on the peak shape of MB for a 1 mL headspace injection. An initial oven temperature of −60°C was chosen to give optimum peak shape. It was also noted that a periodic baseline noise, coinciding with the pressure pulse of the liquid CO_2 oven coolant, was evident during cryogenic isothermal operation

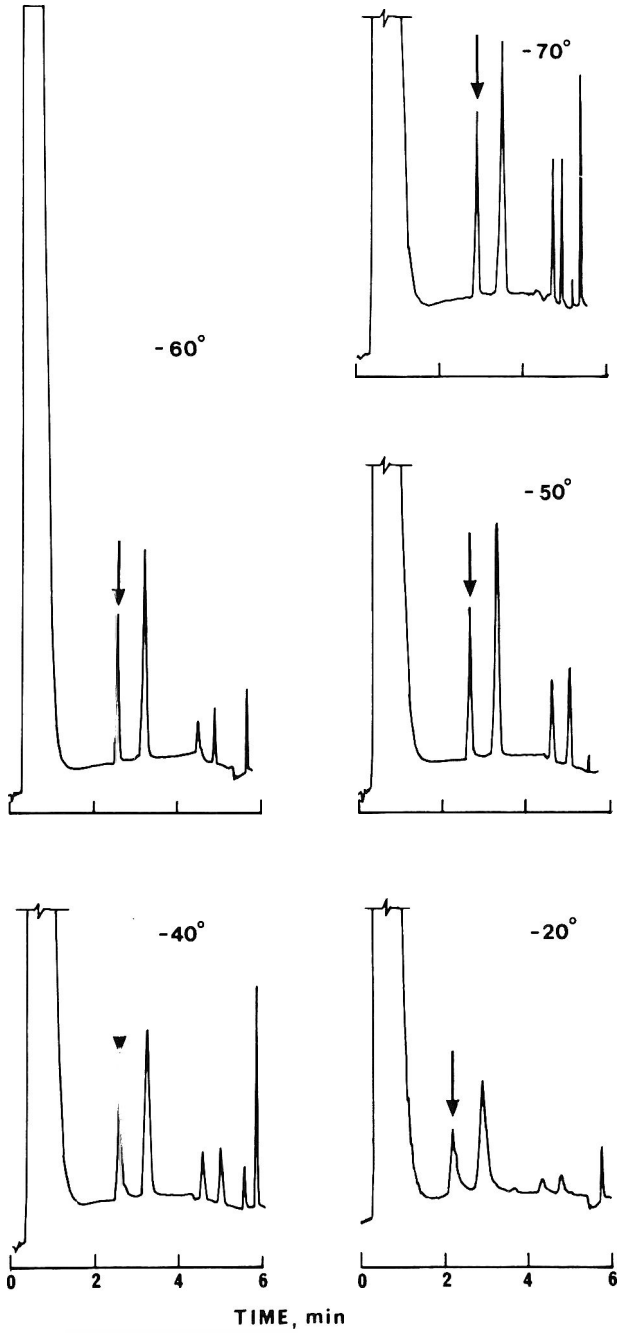

Figure 2. Effect of initial column temperature (°C) on methyl bromide (arrow) peak shape for 1 mL headspace injection.

and temperature programming, if the temperature program was less than about 2°/min. With temperature programming of 5°/min or more, cooling was not needed, and the noise, attributed to the liquid CO_2 cooling pressure pulse, was not present. The on-column injector temperature was kept at 60°C. Reducing this temperature to 0°C or raising it to 100°C did not affect the MB peak.

One of the Health Protection Branch (HPB) regional laboratories demonstrated acceptable chromatography and sensitivity for MB using a different chromatograph, oven temperature, and injector system. They used a 30 m × 0.32 mm id 1 μm film DB-5 column at −25°C for injection and a 2°/min program to −15°C. A splitless injection (1 min closed vent) was also employed. Thus, as long as acceptable peak shape and sensitivity are obtained, other capillary inlet systems, columns, and oven temperatures can be employed. A split injection, however, would reduce method sensitivity.

Several factors affecting the partition of MB into the headspace were investigated using 7.7 ng MB in 20 mL water. For example, 2, 4, and 6 g added anhydrous sodium sulfate were found to increase the MB response by factors of 1.7, 2.6, and 3.1, respectively, compared to no added salt. When 6 g sodium sulfate was added, however, it was difficult to free the magnetic stirring bar from the hydrated salt; therefore, a 4 g addition was chosen. The effect of equilibration temperature on the MB response was also studied using 4 g sodium sulfate and temperatures of 0, 23 (room temperature), and 40°C. A 3-fold increase was found from 0 to 23°C and a 1.2-fold increase was found from 23 to 40°C. For simplicity, room temperature was chosen as the headspace equilibration temperature because temperatures above room temperature would require thermostatted stirring baths. Temperatures above 40° would require a heated syringe to eliminate condensation of water in the syringe during sampling. If the room temperature varies more than a few degrees during the day, additional standards should be run and the calibration curve should be adjusted if required.

Blending or finely dividing the sample using a Polytron homogenizer is required both to obtain a representative sample as well as to release MB from the interior of the sample particle so that the headspace equilibrium will be attained in a reasonable time. In addition, while the blending and sampling are being carried out, no loss of MB by any means should occur. These aspects will be discussed in turn.

The sample being analyzed for MB in the headspace vial must be representative of the whole food from which it was taken. For finely divided foods such as flour, cocoa, and some spices, representative 4 g portions can be taken from the whole food after only sample mixing. For larger particle-size foods, such as grain or peanuts, in which the representative sample is greater than the 4 g required for the headspace analysis, a large representative sample is homogenized or blended and a representative 4 g subsample is taken. This blending or particle-size reduction may be necessary even if the 4 g sample taken is representative of the whole because in headspace analysis the particle size must be reduced so that all MB is released and rapidly equilibrated in the headspace system. Furthermore, in the case of cocoa beans where 80 g are sampled, this blending may be necessary so that the subsample may physically pass through the neck of the flask. Thus, the need for blending or homogenization to produce the 4 g subsample increases as the particle size of the food becomes larger. Finally, for oranges or grapefruits, representative quarters from 4 or more fruit can be blended to give a representative slurry. The need for increasing the size of the blender container as the particle-size increases is self-evident.

For grain, peanuts, and cocoa beans, the homogenizer was required. These foods could not be effectively pulverized or ground in a blender. For foods such as chocolate or cocoa butter, broken and cold 4 g pieces were weighed into the headspace vial containing water and sodium sulfate. The vial was warmed to 40°C, the sample melted, and the vial was cooled with stirring to room temperature and equilibrated. The sample remained finely divided when solidified.

Any study to determine if a representative sample has been obtained requires the use of actual fumigated samples with incurred MB. Table 1 demonstrates good repeatability of the method with both spiked samples as well as with samples after commercial fumigation. The fumigated samples were collected immediately or at intervals after fumigation and were sealed in 1 L glass jars with Teflon-lined screw caps and stored at −20°C. The results in the table demonstrate that

Table 1. Repeatability studies for determination of MB from fumigated (F) and headspace vial spiked (S) samples

Commodity	Sampling[a]	Level, ppb	CV, %
Flour (F)	4 g	0.60	4.77
	4 g	3.8	3.02
	4 g	13.5	4.05
	4 g	72	6.76
Flour (S)	4 g	0.48	5.01
	4 g	9.60	3.15
	4 g	96.0	2.62
Wheat (F)	80 g, 6 aliquots	14.9	3.67
	6 samples of 40 g each	14.9	3.59
Cocoa beans[b] (F)	80 g, 6 aliquots	1460	5.85
	6 samples of 40 g each	1460	10.7
	6 samples of 80 g each	1460	4.91
Parmesan cheese (S)	6 samples of 40 g each	1.92	8.94

[a] n = 6, separate determinations (samples) or slurry aliquots.
[b] 10 g slurry with 10 g water.

the flour samples are sufficiently homogeneous so that a 4 g aliquot is representative of the contents of the 1 L sample jar. For incurred MB, the average CV for the 4 sets of 6 samples each is 4.65% which compares favorably to the 3.59% average for 3 sets of 6 samples each of spiked flour samples spiked over a similar range. For wheat with incurred MB at 14.9 ppb, 6 aliquots, each containing 4 g of homogenized wheat from an 80 g sample gave a CV of 3.63%. Six separate determinations using six 40 g wheat samples and single 4 g aliquots gave a CV of 3.59%. This indicates that 40 g of wheat is representative of the sample jar contents and repeatable results can be obtained.

A similar study involving MB in cocoa beans showed that one may expect a CV of about 5.85% as this result was obtained on 6 aliquots of an 80 g homogenized sample. With 40 g individual samples, the CV was 10.7%, and with 80 g individual samples the CV was 4.91%, which may indicate that 80 g rather than 40 g is necessary to obtain a representative sampling from the sample jar. These results demonstrate that the sample particle size is very important in determining what weight of sample is needed to obtain a representative sample.

The unlikely possibility of equilibration of MB between wheat kernels or cocoa beans, for example, during storage at −20°C in the sample jar, cannot be ruled out. This effect would reduce the sample-to-sample CV value toward that of the aliquot-to-aliquot CV value. With the samples analyzed, the average wheat kernel weighed about 0.03 g and the average cocoa bean weighed about 1 g. These were the only products studied in this manner.

Daft (11) found the kernel-to-kernel coefficients of variation of several fumigants in one sample of corn and one of wheat to be very high and advocated analysis of sample portions in excess of the traditional 25–100 g to determine the truest mean residue level in large volumes of stored grains. Our results tend to support his conclusion. Clearly, a 1 g whole grain sample as proposed by DeVries et al. (10) is inadequate.

To carry out static or equilibrated headspace analysis, an equilibrium of the analyte must be obtained between the condensed phase (liquid and/or solid) and the gas phase. The time required to reach this equilibrium depends on a number of factors including the sample particle size.

The equilibration of MB in an undivided sample, such as whole peanuts, wheat kernels, or cocoa beans, the surrounding aqueous solvent, and the headspace gas will be eventually

attained in a sealed system barring loss by chemical reaction. A similar equilibration is the basis of the cold solvent extraction procedures for fumigant analysis discussed earlier (5–8), and has been shown to take upwards to 48 h. With headspace analysis, equilibration is desired in 1 h or less; thus the sample must be finely divided by blending, grinding, or homogenizing so that all MB is released and rapidly equilibrated. The need to use actual fumigated samples with incurred MB to study various blending procedures and equilibration times so that maximum MB is available to be partitioned into the headspace cannot be overemphasized.

Our initial studies were designed to finely subdivide fumigated peanuts without loss of MB so as to obtain a maximum headspace response for the same weight of peanuts, with all other factors unchanged. For example, cold (−20°C) fumigated peanuts were placed in a cold stainless steel blender jar and the tare weight was taken. Powdered carbon dioxide was added and the peanuts were pulverized. As the remaining carbon dioxide evaporated from the peanuts, the tare weight was approached (+2 g), and the contents of the blender were transferred to a cold glass jar which was sealed and stored at −20°C. The sampling tube for dry foods was used to add 4.0 g pulverized peanuts to the headspace vial containing 25% (w/w) aqueous sodium sulfate and a magnetic stirring bar. The headspace vial containing the peanuts was brought to room temperature over 15 min with stirring. After 1 h equilibrium at room temperature, the headspace was sampled and injected. The inconvenience of solid carbon dioxide, however, required other approaches to be examined. The procedure in which the frozen sample is blended with cold water and ice was adopted. A limited comparison of the carbon dioxide procedure and the cold water–ice blending was carried out and showed that both procedures gave equivalent MB responses.

A comparison of incurred MB levels obtained using whole peanuts or wheat vs the homogenized sample is given in Table 2. The respective unfumigated whole or homogenized sample spiked with aqueous aliquots of MB solutions was used for quantitation. The differences in the MB levels determined on the intact wheat or peanut with a 1 h equilibration compared to those levels determined on the homogenized samples results from 2 contributing factors. First, with the wheat, the MB response from the homogenized fumigated samples ranged from about 2.2 to 7.0 times that of the intact sample. This is attributed to the release and rapid equilibration of both surface and subsurface MB following homogenization compared to the slow equilibration and release of MB with the intact sample. Second, when unfumigated wheat samples were spiked with aqueous MB aliquots the MB response of the homogenized sample was 0.80 to 0.91 times that of the intact sample. Again, an equilibrium is attained with the homogenized sample and not with the intact sample. The resultant effect is that calculated MB levels for the fumigated wheat were about 3.3 to 8.8 times greater when determined on the homogenized wheat than when determined on the intact sample. For peanuts, a similar study gave calculated results 3.7 and 5.8 times higher for the homogenized sample. This discrepancy resulted mainly from the chromatographic responses of the spiked homogenized samples being 0.36 to 0.50 times that of the intact samples. The responses of the homogenized fumigated samples were about 1.4 times that of the intact peanuts.

Clearly, any analytical data determined using an equilibrium headspace procedure and intact samples (with spiked blank samples), such as described previously (10), should be compared to the results determined on the homogenized fumigated sample and appropriate standard to ensure the proper equilibrium is attained.

Table 2. Comparison of headspace results[a] for MB in fumigated wheat and peanuts calculated from whole or homogenized sample with corresponding standard

Commodity	Homogenized, ppb	Whole, ppb
Wheat	281	84.3
	150	35.5
	14.9	3.1
	2.2	0.25
Peanuts	20200	5500
	26100	4500

[a] 1 h equilibrium with stirring at room temperature for 3.0 g wheat or peanuts in aqueous sodium sulfate.

To determine the equilibrium time required for homogenized or finely divided peanuts, and by analogy, for other homogenized products, individual aliquots of homogenized fumigated and spiked blank peanuts were stirred and sampled periodically. Plots of MB peak height vs time (0.25, 0.5, 1.0, 2.0, 4.0, 6.0, and 6.5 h) showed the spiked sample response initially decreased and the fumigated sample response increased with time, both leveling off at 1 h, after which both responses decreased about 10% over the next 5.5 h. This shows that 1 h is sufficient for equilibrium to be reached both with incurred and fortified samples.

Magnetic stirring was considered essential to help dissolve the sodium sulfate, to help equilibrate the flask rapidly to room temperature in about 15 min, and to obtain the equilibration at room temperature in 1 h.

The final requirement, that MB must not be lost by volatilization or chemical reaction during the homogenization or transferring of the sample slurry, was readily demonstrated by a simple study: 40 g unfumigated wheat, 85 g ice, and 75 g water in each of two 250 mL Erlenmeyer flasks were spiked separately below the surface with 1 mL aqueous standards containing either 38.5 ng (0.95 ppb) or 192.5 ng (4.8 ppb) MB. The contents of the flasks were homogenized for 3.5 min, and duplicate 20 g (4 g wheat) aliquots were analyzed as described in the method. The homogenization was repeated with 40 g wheat, 80 g ice, and 70 g water but without spiking; 19 g aliquots (4 g wheat) were added to each of 4 headspace vials each containing 4 g sodium sulfate and a magnetic stirring bar. The vials were chilled in water–ice, spiked in duplicate below the surface with 1 mL aqueous standards containing either 3.85 or 19.25 ng MB, quickly sealed after each fortification, and analyzed as described in the method.

The headspace responses of the homogenized bulk-spiked samples were compared to those of the individually headspace vial spiked samples to determine the loss of MB attributed to volatilization during homogenization or slurry transfer. Recoveries of 89.0% at 0.95 ppb and 95.6% at 4.8 ppb demonstrate that little loss of MB is due to these factors.

The sensitivity of the headspace procedure described above for determining MB in a food compared to a direct injection of a solution obtained following cold solvent extraction can be estimated from the following considerations. With capillary column chromatography an on-column 1 μL injection of 30 pg of MB in isopentane gives about 10% full scale deflection (FSD) at 16×. A 1 mL headspace sampling over an aqueous solution containing ca 1.2 ng of MB in 20 mL water with 4 g added sodium sulfate in a 30 mL headspace vial (actual volume ca 37 mL) also gives about 10% FSD at 16×. Thus, a headspace of 17 mL containing 30 pg/mL represents a 40% partition into the headspace. With a food containing 1 ppb

Table 3. Relationship of food lipid content and headspace response for 7.7 ng MB with 4 g food and calculated sensitivity

Food	Peak ht, mm at 16X	5% FSD (ppb)	Lipid,[a] %
Water	124	0.15	0
Raisins	119	0.15	0.2–0.5
Cocoa	104	0.18	2
Flour, wheat	97	0.19	1
Peppercorns	88	0.21	7
Parmesan cheese	56	0.33	26–28
Celery seeds	49	0.37	NA[b]
Chocolate (unsweet.)	47	0.39	53
Cocoa beans	41	0.45	50–57
Sesame seeds	36	0.51	49–55
Cocoa butter	34	0.54	100
Pumpkin seeds	32	0.57	47
Peanuts	28	0.65	45

[a] Taken from ref. 12.
[b] Value not available.

MB, 4 g food (4 ng MB) in aqueous solution would partition 1.6 ng into the headspace, if one ignores the effect of food on the partition ratio. A 1 mL headspace sampling, therefore, contains almost 100 pg (~30% FSD). A cold solvent extraction of 1 part of the above food with as little as 2 parts solvent (13) gives a sample concentration of 0.5 g/mL and a MB concentration of 0.5 ng/mL. A 1 μL injection contains 0.5 pg, well below the detection threshold. This supports the contention that the headspace procedure for MB is much more sensitive than any solvent extraction method.

The presence of food, however, will decrease the headspace response as shown in Table 3. These results suggest an inverse relationship of MB headspace response and lipid content—although other factors may be operative. The quantitation level, if defined as 10 times the noise level or ca 5% FSD would range from 0.15 to 0.65 ppb. Because of this range of responses, a separate calibration curve must be determined and used for each food. It was observed that dilution of standards and spiking of foods for calibration purposes was best carried out with 1 mL transfer of the ice-cold standard solutions. Initially, μL quantities were used for spiking; however, the resulting calibration curves were inferior to those obtained using ≥ 1 mL aliquots.

A number of spices, seeds, nuts, and dried fruits and vegetables including samples of celery, mustard, sesame, coriander, pumpkin, and sunflower seeds, cloves, peppercorns, dates, figs, prunes, raisins, beans, minced onion, a vegetable mix, walnuts, and peanuts were purchased locally in an attempt to obtain foods with incurred MB residues. Of 30 samples, only a sample of pumpkin seeds was found to contain MB, and only at ca 3 ppb; 51 chocolate and grain-based products were also analyzed and found to be negative for MB. Another laboratory, using the described procedure but a different capillary column and chromatographic conditions, analyzed 111 samples of selected food products and did not detect any MB. Samples of foods known to be treated with MB were also analyzed. Of 60 samples, 16 contained residues > 1 ppb and 5 contained > 100 ppb MB. These samples included dried currants (2900 ppb), chocolate-covered nuts (660 and 190 ppb), and rice (2300 ppb) and corn (210 ppb) flour. These results were confirmed by mass spectrometry.

With all samples, it was noted that there was little or no interference in the area of MB. Sample chromatograms illustrating several different foods with both spiked and incurred MB are shown in Figure 3.

Incurred residues can be confirmed by various procedures.

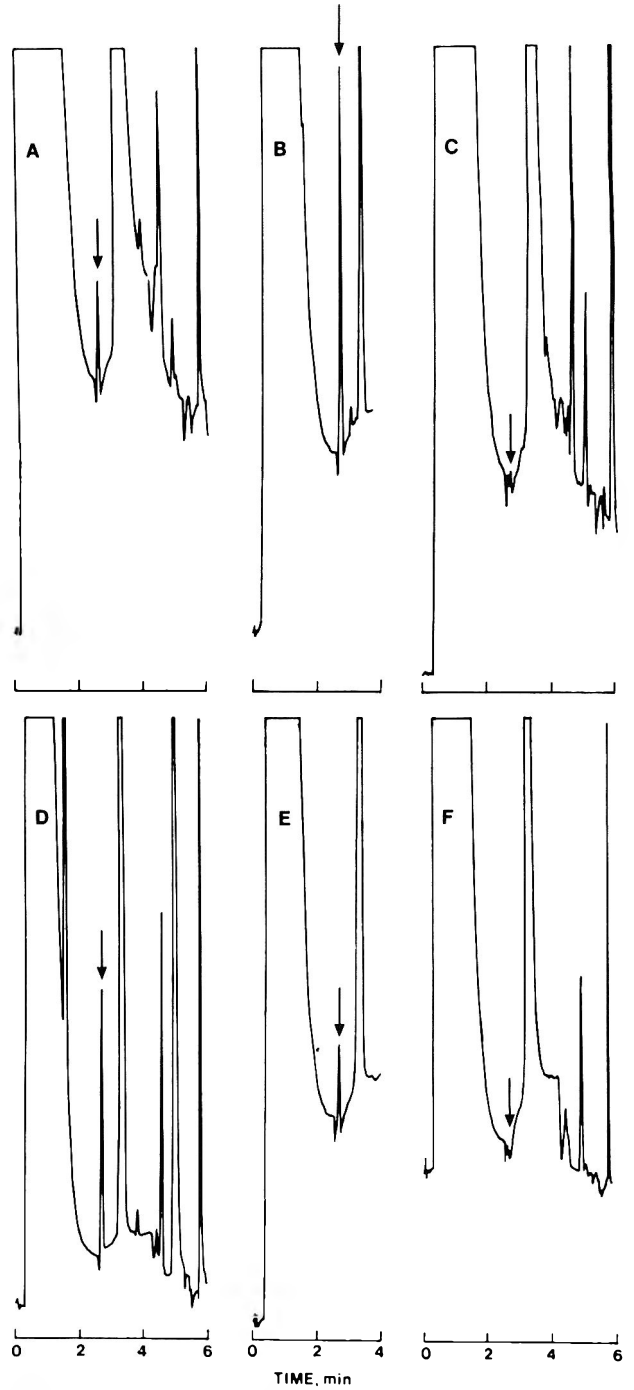

Figure 3. Representative chromatograms of methyl bromide (MB, arrow) in foods: A, flour, 0.60 ppb (incurred); B, raisins, 1.9 ppb (spike); C, cocoa, fumigated cocoa beans, MB not detected; D, wheat, 2.5 ppb (incurred); E, peanuts, 1.9 ppb (spike); and F, parmesan cheese, fumigated, MB not detected.

In our laboratory the Hall Model 700A electrolytic conductivity detector operating in the reductive halogen mode has been used for confirmation of MB levels in flour. In our limited investigation, sensitivity about one half that of the electron capture detector was obtained. Whereas the calibration curves with the electron capture show a slight decrease in response/ng as the analyte concentration increases, with the Hall detector the response/ng increases as the concentration increases. Another laboratory, using the described procedure but with a DB-1 capillary column, confirmed by mass spectrometry the identity and level of MB using selected ion monitoring of the molecular ions at 94 and 96 m/z, the molecular ions of the 2 bromine isotopes of methyl bromide.

The major advantage (and difference) of the described procedure over other headspace procedures is the food particle size reduction by blending or homogenization of the cold or frozen sample with ice and cold water with only minimal loss of MB. This permits a rapid 1 h equilibration in the headspace vial of both MB trapped within the food as well as MB standard added to blank foods when calibration data are developed. The blending or homogenization of larger samples also allows 4 g representative and repetitive food samples in 20 g slurry to be taken for headspace equilibration and analysis. This comminution procedure should also be applicable to other fumigants with volatilities similar to or less than that of MB. A description of a gas-tight syringe adaptation to permit manual headspace sampling of any size of conventional headspace vial and on-column capillary injection is also given.

The advantages of the headspace procedure described above for the determination of MB in foods clearly outweigh those of cold solvent extraction of the whole grain. With headspace, nonvolatile material is not introduced into the chromatographic column or injector body; the time from sample acquisition to analytical result can be less than 2 h, including sample homogenization, a 1 h equilibration, and short chromatographic run; the method is sensitive with detection limits typically 0.15 to 0.65 ppb; and, finally, only simple and inexpensive equipment, apart from the gas chromatograph, is required. In contrast, with the solvent extraction procedure, nonvolatile or late-eluting food components such as lipids may be co-injected with the volatile fumigant; the time required to obtain an analytical result is mainly determined by the extraction period, usually 24 h (8) or possibly longer (7); and the method is not as sensitive as headspace due to the low sample concentration (≤ 0.5 g/mL) of the injected solvent. The equipment required is also inexpensive and less complex than that for headspace.

There are 2 major disadvantages with the headspace procedure. First, duplicate injections from the same vial are not possible. Once the integrity of the septum has been disrupted, any duplicate sampling would be suspect. Duplicate injections require duplicate samples and headspace vials. Second, the headspace partition varies with the food analyzed; for quantitation, a separate calibration curve is required for each food. This could be overcome, however, if an electron-capturing internal standard, similar in volatility and polarity to MB, was added to each headspace vial. Qualitative screening, however, can be carried out without a calibration curve.

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REFERENCE STANDARDS

Preparation of Reference Water and Soil Samples for Performance Evaluation of Volatile Organic Analysis

MICHAEL P. MASKARINEC, LYNNE H. JOHNSON, and CHARLES K. BAYNE
Oak Ridge National Laboratory, Analytical Chemistry Division, PO Box 2008, Oak Ridge, TN 37831-6120

Methodology was developed to reproducibly prepare performance evaluation materials for volatile organics analysis in soils and waters. Tedlar gas sampling bags are used to prepare the volatile organics spike solutions. The bags allow large volumes of sample or spike solution to be prepared to a high degree of homogeneity while using less methanol. Preparation of a large volume of sample or spike solution allows for increased accuracy of fortification. The accuracy is generally ±20% or better, and the precision is generally ±10% or better for water samples. The precision for preparation of soil samples is also good, but the accuracy suffers from variable recovery efficiencies from the soils. Most volatile organics were well preserved in 3 water samples by storage at refrigerator temperature for 14 days.

Demand for analyses of environmental samples for volatile organic compounds has dramatically increased in recent years. This is due to increased public awareness of the growing problem of hazardous waste disposal and the abundance of hazardous waste dump sites nationwide which require remedial action. The need for hazardous waste cleanup and the methodology required for establishing environmental sample banks has brought about the simultaneous need for analytical support. Some of the most commonly identified contaminants include volatile organics.

To provide for consistent results from analytical laboratories nationwide, the U.S. Environmental Protection Agency (EPA) has issued various analytical methods in the *Federal Register* to standardize analyses. Among the quality assurance needs in these methods is the need for reference samples. Such samples enable interlaboratory comparisons to be made. Because of difficulties encountered in the preparation of reference samples for volatile organics, laboratories commonly add a methanolic solution of these compounds to blank water samples immediately prior to performing an analysis. This situation precludes the use of blind performance evaluation samples. The objective of the present work is to provide methodology capable of producing performance evaluation materials for volatile organics in water and soil that are truly blind, and therefore allow a more relevant assessment of the precision and accuracy of the analytical methods.

Two criteria need to be met by performance evaluation samples. These samples need to be "real", i.e., they should closely simulate the composition of actual samples. This is due to the need for reference samples to be blind samples. Also, these samples need to be of defined stability. This is necessary because of sample preparation and transfer time to the analysis sites, and sample analysis requirements at the analytical laboratory. These 2 criteria often conflict. For example, previous work by Friedman, Schroder, and Brooks (1), in which methanol was added to samples spiked with volatile organic compounds to check the preservative effect of such an addition, found no improvement in sample stability. However, the added methanol would have easily been noted by an experienced analyst, thus identifying the sample as a reference sample. Stability is a concern with volatile organic samples not only because the compounds of interest can be lost through volatilization, but also because some of these compounds are susceptible to chemical and biological degradation.

Current EPA Contract Laboratory Protocol (CLP) guidelines dictate a maximum holding time prior to analysis for volatile organics analysis (VOA) samples of 10 days. This is a very stringent limit for most analytical laboratories because of instrument maintenance requirements, quality assurance data review, and data processing requirements. Moreover, the stability of analytes during long-term storage is of vital concern in establishing methodology for environmental sample banking (2). Therefore, it would be useful to have a database for samples containing volatile organic compounds, which established the sample stability for these compounds in many sample matrixes. Such a database would open the possibility of extending the maximum holding time limits for VOA samples, but it could only be generated if methods were available for the preparation of multiple sample aliquots. The present study addressed the problems encountered in preparing soil and water samples that contain reproducible levels of volatile organic compounds.

Experimental

Materials

The sample storage vials used were 40 mL borosilicate glass vials with Teflon-faced silicone septa and screw caps with holes (purchased from Shamrock Glass Co., Cat. No. 6-06K). These vials were received fully assembled and precleaned according to EPA 40 CFR 136 and EPA 40 CFR 141 regulations. One L Tedlar air sampling bags had dual stainless steel fittings (hose/valve fitting and replaceable septum) (SKC, Inc., Cat. No. 231-01). Methanol and acetonitrile were distilled-in-glass grade (Burdick & Jackson).

Three water and 3 soil sample matrixes were used for this study. The waters were reagent grade water (water 1) (Burdick & Jackson), ground water (water 2), and surface water (water 3). The ground water was drawn from Well No. 1 at the Oak Ridge National Laboratory (ORNL) Aquatic Ecology Facility (well depth: 205 ft; static water level below ground level: 30 ft). Surface water was taken from the headwaters of White Oak Creek. The soils were U.S. Army Toxic and Hazardous Materials Agency (USATHAMA) reference soil (soil 1), Tennessee 0800 (obtained from Oak Ridge Department of Energy Reservation) soil (soil 2), and Mississippi topsoil (soil 3). Soil 1 was obtained from USATHAMA and soils 2 and 3 were obtained from the Environmental Sciences Division, ORNL.

All target compounds either were obtained from EPA Quality Assurance Materials Bank (Research Triangle Park, NC) or were equivalent purity and obtained commercially (Burdick & Jackson). The following analytes were included in the study: bromomethane, chloroethane, methylene chlo-

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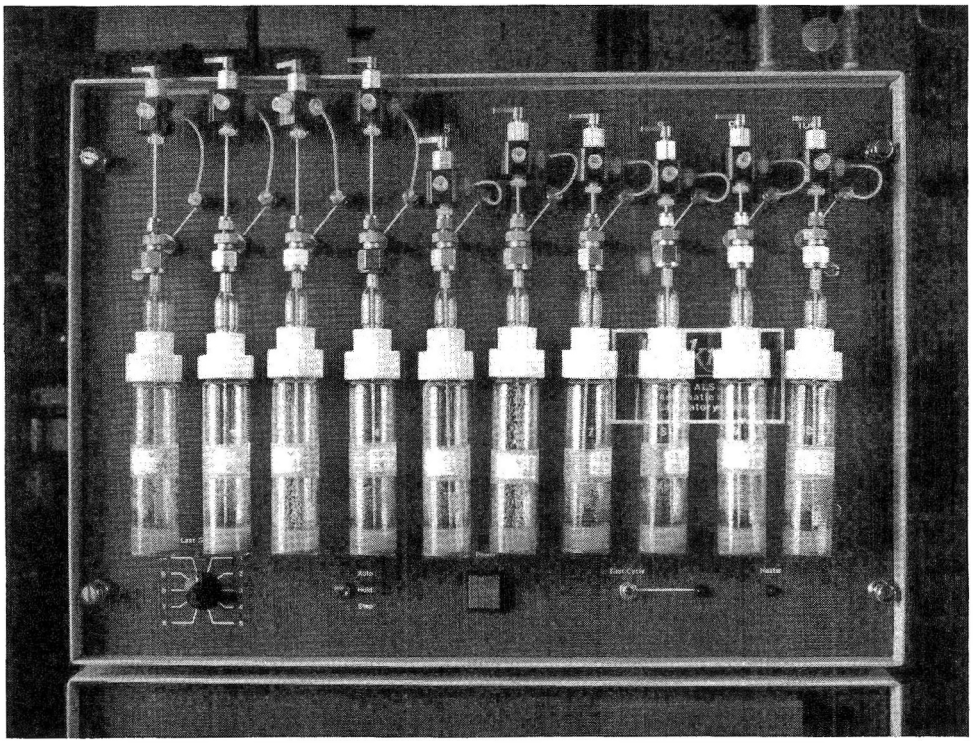

Figure 1. Experimental purge and trap arrangement for soil samples.

ride, 1,1-dichloroethene, 1,1-dichloroethane, chloroform, carbon tetrachloride, 1,2-dichloropropane, trichloroethene, benzene, 1,1,2-trichloroethane, bromoform, 1,1,2,2-tetrachloroethane, tetrachloroethene, toluene, chlorobenzene, ethylbenzene, styrene, and *o*-xylene.

Method

Water samples.—The desired water was dispensed into a 1 L Tedlar gas sampling bag (3, 4). The water was allowed to degas for 2 days, and the gas was removed from the bag. Target compounds were received from the aforementioned sources as methanolic solutions of 1800–2300 μg volatiles/mL methanol. Appropriate volumes of each stock volatile organic solution were introduced through the septum port via gas-tight syringes. The contents of the Tedlar bag were mixed thoroughly by hand agitation for 3 min after which the bags were allowed to sit for 30 min. After mixing, aliquots of the sample were transferred to the 40 mL vials by gravity flow. Teflon tubing (¼ × 6 in.) was used to allow the vial to be filled from the bottom upward, preventing mixing of the water with air. Each bottle was completely filled with sample so that no headspace would remain after the bottle was sealed. Each bottle was sealed immediately with a Teflon-faced septum and screw cap with hole, and stored at the appropriate temperature (4 and 25° C).

Soil samples.—Five g soil was added to a 40 mL vial. Twenty to 25% (volume/weight) reagent grade water was added to partially wet each soil sample. Each vial was sealed with a Teflon-faced septum and screw cap with hole. Each vial was mixed on a Vortex mixer for about 30 s and allowed to sit at room temperature in the dark for 3 days. This was done to initiate bacterial activity in the soil and allow the activity to come to equilibrium (personal communication, B. T. Walton, Environmental Science Division, ORNL, 1986). The spike solution of volatiles was prepared in a Tedlar bag as described for water samples. The spike solution was added to each soil sample at the rate of 0.20–0.25 mL aqueous volatiles/g soil. At this point, the soil sample was 80–100% saturated with water. Each vial was immediately capped, and the contents were mixed on a Vortex mixer for about 30 s and stored or analyzed as appropriate.

Analysis

All volatile organic analyses were performed by purge-and-trap gas chromatography with mass spectroscopic detection (GC/MS) (5) approximately according to standard EPA Contract Laboratory Program (CLP) methods (6). In this procedure, an inert gas is bubbled through the sample, removing volatile compounds from the aqueous phase and carrying them to a sorbent column. Volatiles are trapped on this column until it is heated and backflushed, desorbing the purged compounds onto a gas chromatographic column. Temperature-programming of the column oven permits separation of the volatiles mixture; individual components are then detected and identified by mass spectroscopy.

Soil samples (with added reagent water) were purged directly from the 40 mL vials by use of Teflon couplings machined in-house for this study. This was done to allow an assessment of the actual content of the vials without the complicating factor of sample weighing and transfer. A diagram of this experimental arrangement for soil samples is shown in Figure 1.

Results and Discussion

Precision and Accuracy

There are 2 possible approaches to the preparation of multiple aliquots of performance evaluation materials. The sample vials can be prepared and individually spiked, or a large volume of sample can be prepared and aliquots can be taken. In most cases, the second approach is preferred, since this approach should provide for greater sample preparation precision. However, for the volatile organic compounds, the possibility of changing sample composition during dispensing

Table 1. Repeatability of VOA spiking into individual VOA vials (μg volatiles/L water)[a]

Compound	Rep. 1	Rep. 2	Rep. 3
Carbon tetrachloride	30	21	21
Chlorobenzene	29	20	19
1,1-Dichloroethane	30	20	20
1,1,2-Trichloroethane	35	25	23
Chloroform	27	18	19
1,1-Dichloroethene	33	22	22
1,2-Dichloropropane	34	24	24
Methylene chloride	33	20	19
Chlorodibromomethane	27	18	17
Trichloroethene	17	12	12

[a] Added concentration: 30 ± 6 μg/L.

Table 2. Stability of aqueous volatiles in Tedlar bags at 4°C[a]

	Day 0		Day 3	
Compound	Mean, μg/L	RSD, %	Mean, μg/L	RSD, %
Carbon tetrachloride	33.8	1.3	33.8	1.7
Chlorobenzene	32.5	1.0	33.3	1.0
1,1-Dichloroethane	34.3	1.5	30.0	1.7
1,1,2-Trichloroethane	45.8	1.5	52.0	5.0
Chloroform	52.3	1.5	84.0	2.6
1,1-Dichloroethene	29.8	2.2	26.3	2.0
1,2-Dichloropropane	41.5	2.1	45.8	1.7
Methylene chloride	45.5	1.3	57.0	1.4
Chlorodibromomethane	42.3	1.5	47.8	2.2
Trichloroethene	22.3	1.3	28.0	0.0

[a] Added concentration: 40 ± 8 μg/L; n = 4.

of aliquots complicates the situation. A preliminary study was performed to evaluate the differences between these approaches for water samples. A systematic error was immediately noticed between vials, and the amount of methanol had to be increased in proportion to the volatile organic analytes (Table 1). Large amounts of methanol create problems in the analysis. The amount must be kept below 50 μL/40 mL.

The use of the Tedlar gas sampling bags provided several advantages. First, large volumes of sample could be prepared using minimal amounts of methanol. Second, vial-to-vial variability was reduced. Third, the sample could be successfully stored in the bag (Table 2). Fourth, the accuracy of the concentration of the analytes in the sample was improved due to the relatively larger volume of spiking solution. Finally, the bag allowed the filling of the individual sample vials with no introduction of headspace and minimal mixing of the aliquots with air during filling of the vials.

Data from the analysis for 17 volatile organics in 3 water samples on day zero are given in Table 3. With a few exceptions, the standard deviations from 4 replicates for each data point are approximately 10% or less of the mean value. It is useful to look at each individual water sample at a given concentration level to check the average of the data for all of the compounds. Table 3 also gives the average of the values for all compounds, for each water sample and concentration level. The target concentrations were 50 μg/L for the low concentration level and 500 μg/L for the high concentration level. All of the averages of the values for a given sample and concentration level are within 15% of the target concentration, with standard deviations of around 20% or less.

Data from the analysis for the same volatile organics in 3 soil samples on day zero are given in Table 4. The target concentration for these samples was 100 μg/kg. The data indicated actual concentrations in the range of 14 to 118 μg/kg with standard deviations of less than 25%. Although the accuracy is not as good as with the water samples, there is the additional complication of variable recovery of the analytes using the analytical method. Such variability is evidenced by the surrogate and matrix spike recovery limits specified in the EPA CLP quality assurance requirements (6), which have much wider limits for soil than for water. However, the precision is acceptable for the purpose of performance evalu-

Table 3. Volatile organic compounds in water samples[a]

	Water 1 low level		Water 1 high level		Water 2 low level		Water 2 high level		Water 3 low level		Water 3 high level	
Compound	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Methylene chloride	53.1	3.5	464.1	31.3	66.4	1.5	396.1	111.3	69.9	3.7	544.0	38.8
1,1-Dichloroethene	57.6	3.2	472.9	17.5	61.3	1.7	383.4	121.1	53.2	4.6	408.9	61.3
1,1-Dichloroethane	63.9	2.7	514.7	18.1	66.2	1.6	388.6	111.3	64.7	3.4	593.8	59.5
Chloroform	59.6	1.8	488.8	25.0	61.0	1.4	385.5	114.3	69.5	4.1	570.3	46.7
Carbon tetrachloride	50.7	3.8	445.9	27.2	56.2	1.0	551.1	39.4	46.8	2.6	681.8	62.4
1,2-Dichloropropane	49.7	3.0	421.0	13.2	34.4	0.3	589.7	64.5	55.1	2.3	553.7	79.2
Trichloroethene	56.7	3.9	287.0	1.4	107.9	2.9	570.3	63.2	60.0	3.6	505.7	61.0
Benzene	49.6	2.7	400.2	11.1	54.6	0.2	569.7	50.2	55.5	2.3	479.7	34.7
1,1,2-Trichloroethane	47.3	1.7	440.5	17.8	55.4	1.0	584.4	74.9	59.4	2.1	649.5	150.1
Bromoform	33.9	3.5	619.3	47.2	49.6	0.1	583.3	77.9	54.8	2.2	766.1	178.2
1,1,2,2-Tetrachloroethane	41.8	1.3	531.3	49.1	48.5	6.0	544.0	36.5	54.2	5.5	797.3	127.1
Tetrachloroethene	49.8	3.1	369.7	19.1	52.6	1.7	530.6	40.5	57.0	4.3	398.1	47.4
Toluene	49.7	3.1	383.0	19.4	54.1	0.8	550.0	34.4	54.3	3.3	462.3	35.0
Chlorobenzene	51.6	2.6	381.7	21.7	55.4	1.1	547.4	36.5	55.9	3.6	476.9	33.9
Ethylbenzene	48.6	1.5	378.0	23.8	51.2	1.1	532.2	43.7	52.1	4.1	416.8	42.2
Styrene	52.5	2.4	394.1	24.4	63.3	1.8	535.2	41.2	57.7	3.5	486.1	28.7
o-Xylene	53.7	2.7	397.5	23.4	53.0	0.9	531.4	36.0	58.3	3.9	473.6	34.2
Mean	51.2		434.7		58.3		516.1		57.6		545.0	
SD	6.6		74.5		14.4		73.1		5.8		115.7	
RSD, %	12.8		17.1		24.8		14.2		10.0		21.2	

[a] Concentration added: 50 ± 10 μg/L (low level); 500 ± 100 μg/L (high level). Found, μg/L (n = 4).

Table 4. Volatile organic compounds in 3 soils[a]

Compounds	Soil 1		Soil 2		Soil 3	
	Mean	SD	Mean	SD	Mean	SD
Bromomethane	62	10	44	9	92	3
Chloroethane	81	18	80	6	98	4
Methylene chloride	67	8	72	7	118	11
1,1-Dichloroethene	84	10	88	17	93	3
1,1-Dichloroethane	86	11	91	15	102	0
Chloroform	73	7	79	12	92	2
Carbon tetrachloride	84	8	86	21	95	4
1,2-Dichloropropane	71	10	82	16	96	5
Trichloroethene	73	10	69	17	79	3
Benzene	67	5	69	15	69	2
1,1,2-Trichloroethane	53	6	60	6	80	7
Bromoform	41	5	29	2	50	4
1,1,2,2-Tetrachloroethane	42	6	40	3	76	2
Tetrachloroethene	64	9	56	13	78	5
Toluene	70	8	49	7	64	2
Chlorobenzene	52	6	35	4	51	1
Ethylbenzene	67	7	43	6	58	1
Styrene	45	3	16	3	14	1
o-Xylene	65	6	39	3	57	3

[a] Concentration added: 100 ± 10 μg/kg. Found, μg/kg (n = 4).

ation. Recovery of the highly volatile compounds bromomethane and chloroethane indicates that analyte losses to volatilization were successfully restricted by sample preparation and storage methods.

Reproducibility

Table 5 presents mean values derived by averaging day zero results for a particular compound from all 3 volatile organics in water samples with a given target concentration. All compounds except trichloroethene had a standard deviation of less than 20%. Indeed, for many compounds at low concentration, the relative standard deviation was less than 10%. Table 5 clearly indicates a high degree of reproducibility can be obtained for volatile organics in water samples by the methodology of sample preparation and analysis presented in this paper.

Table 5. Statistical data from analysis of low and high concentration levels of 3 water samples[a]

Compound	Low level			High level		
	Mean	SD	RSD, %	Mean	SD	RSD, %
Methylene chloride	63.1	7.2	11.5	468.1	60.5	12.9
1,1-Dichloroethene	57.4	3.3	5.8	421.7	37.7	8.9
1,1-Dichloroethane	64.9	0.9	1.4	499.0	84.5	16.9
Chloroform	63.4	4.4	6.9	481.5	75.6	15.7
Carbon tetrachloride	51.2	3.8	7.5	559.6	96.5	17.2
1,2-Dichloropropane	46.4	8.8	18.9	521.5	72.6	13.9
Trichloroethene	74.9	23.4	31.3	454.3	121.2	26.7
Benzene	53.2	2.6	4.9	483.2	69.2	14.3
1,1,2-Trichloroethane	54.0	5.0	9.3	558.1	87.3	15.6
Bromoform	46.1	8.9	19.3	656.2	79.1	12.0
1,1,2,2-Tetrachloroethane	48.1	5.1	10.5	624.2	122.5	19.6
Tetrachloroethene	53.1	3.0	5.6	432.8	70.1	16.2
Toluene	52.7	2.1	4.0	465.1	68.2	14.7
Chlorobenzene	54.3	1.9	3.5	468.7	67.9	14.5
Ethylbenzene	50.6	1.5	2.9	442.3	65.5	14.8
Styrene	57.8	4.4	7.7	471.8	58.5	12.4
o-Xylene	55.0	2.4	4.3	467.5	54.8	11.7

[a] Found, μg/L (n = 3).

Table 6. Statistical data from analysis of 3 soil samples

Compounds[a]	Mean	SD	RSD, %
Bromomethane	66	20	30
Chloroethane	86	9	10
Methylene chloride	86	23	27
1,1-Dichloroethene	89	4	4
1,1-Dichloroethane	93	7	8
Chloroform	81	8	10
Carbon tetrachloride	88	5	5
1,2-Dichloropropane	83	10	12
Trichloroethene	73	4	6
Benzene	68	1	1
1,1,2-Trichloroethane	64	11	17
Bromoform	40	9	21
1,1,2,2-Tetrachloroethane	53	16	31
Tetrachloroethene	66	9	13
Toluene	61	9	14
Chlorobenzene	46	8	17
Ethylbenzene	56	10	17
Styrene	25	14	57
o-Xylene	54	11	20

[a] Initial spike level, 100 μg/kg.

Table 6 presents similar data for the soil samples. Again, the precision and accuracy are not as good as for the water samples, but it is difficult to distinguish sample variability from method variability. In fact, it could be assumed that since the analytes are added to the soil samples in a manner identical to that for the water samples, precision and accuracy of the sample preparation should be roughly equivalent. The increased variability in the results for soil samples is probably due to method variability.

Stability

The study of the stability of these compounds is the subject of a second report, and the results will be summarized here only to the extent that they affect the production of performance evaluation samples. Most of the target compounds remained at or close to their day zero concentration on day 14 for storage at both 25 and 4°C. Exceptions to this are 1,1,2,2-tetrachloroethane and styrene. These 2 compounds degraded significantly during storage at 25°C. The loss of 1,1,2,2-tetrachloroethane was accompanied by a simultaneous increase in the concentration of trichloroethene, indicating that the decay mechanism of 1,1,2,2-tetrachloroethane may be a dehydrohalogenation process. Degradation of styrene was probably due to chemical oxidation. These results indicate that many of the commonly found volatile organics are suitable for use as performance evaluation analytes using these methods.

Conclusions

The results of this study show that volatile organic performance evaluation samples can be prepared to an approximate concentration with reasonable precision in both soil and water matrixes. Furthermore, it is shown in water that most of the volatile organics of interest are stable at refrigerator temperature for a time sufficient to allow distribution and analysis. This should allow reference samples for volatile organics in water to be prepared at a central laboratory and distributed to participating analytical laboratories. Studies of interlaboratory performance of this method on blind sam-

ples can now be performed for the first time. As the stability of the samples is verified, intralaboratory performance over long periods of time will also be possible for the first time.

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Comparison of Volatile Acid Number Test with Enzymatic Acetic Acid Assay for Assessment of Seafood Quality

HELEN T. McCARTHY, P. CHRISTOPHER ELLIS, MARY LOU SILVA, and BARBARA MILLS
Rhode Island Department of Health, Division of Laboratories, 50 Orms St, Providence, RI 02904

Volatile acid number, an official AOAC method for assessing seafood quality, was compared with an enzymatic method for measuring acetic acid. Thirty-eight samples of fresh fish and 23 samples of canned fish were evaluated. The data showed high positive correlations between volatile acid number and enzymatic acetic acid methods; the correlation coefficient was 0.98 for fresh fish and 0.95 for canned fish. Positive correlation coefficients of 0.93 and 0.96 were also associated with samples of fish divided into 2 groups on the basis of acceptable or unacceptable quality, respectively. There was no significant difference between the means for the volatile acid number and enzymatic acetic acid procedures at the 5% level of significance. Decomposition studies on cod and mackerel, representing a lean and fatty fish, respectively, were monitored over 9 days by using the following analyses: volatile acid number, acetic acid, total volatile bases, and thiobarbituric acid. Results of the decomposition studies showed that enzymatic acetic acid levels closely paralleled volatile acid number values in both lean and fatty fish. Total volatile bases was a reliable supplemental measure for evaluating lean seafood quality, whereas thiobarbituric acid was more appropriate for further assessment of fatty fish quality.

Decomposition of fish is a progressive proteolysis of muscle tissue brought about primarily by the action of microorganisms and, to a lesser extent, by autolytic enzymes (1).

Assessment of the overall quality of seafood and seafood products is a major concern for regulatory food laboratories. Sensory evaluation has long been recognized as important in determining the quality of seafoods, but the establishment of effective organoleptic panels can be costly and results are ultimately limited by the evaluators' subjectivity. Various chemical tests are also available to measure seafood wholesomeness. Chemical markers such as indole in shrimp (2, 3), histamines in tuna and in other fish belonging to the family Scrombridae (4), and trimethylamine-nitrogen (5) for various marine fish such as cod and mackerel are useful and specific indicators of seafood quality. Among the more general chemical markers of seafood spoilage are: total volatile bases (TVB), a measure of protein breakdown and volatile amines (particularly trimethylamine originating from trimethylamine oxide); thiobarbituric acid (TBA), a measure of malonaldehyde from lipid breakdown; and volatile acid number (VAN), a measure of volatile acids resulting from bacterial proteolysis.

Procedures employed for quality assessment should be applicable to routine testing of large quantities of fish and should take into consideration such factors as specificity, cost, time to perform the assay, and necessary expertise of the analyst. Although methods for assessing seafood quality and wholesomeness are available, few methods meet all these considerations. In addition, since changes occurring in fish during decomposition are known to be very complex, a single chemical index may not be a reliable indicator for a particular sample of fish. Therefore, several different chemical methods of assessment along with an organoleptic evaluation should be employed in evaluating seafood quality.

Volatile acid number, an official AOAC method of analysis (5), is a measure of the lower members of the fatty acid series including acetic, formic, propionic, and butyric acids. Volatile acids higher than acetic acid are not found in pelagic U.S. east coast fish or in canned fish (6, 7). Formic and acetic are the acids most frequently detected, with acetic acid predominating (1, 6, 7). Seafood spoilage leads to an increase in overall volatiles (8), and increase of acetic acid is very pronounced throughout the spoilage process. Formic acid, in contrast to acetic acid, shows trace amounts initially and increases only after decomposition is noticeable organoleptically (7).

Although the VAN procedure is an accurate measure of volatile acids, the procedure itself is not applicable to routine testing of large numbers of samples. The method is also highly sensitive to small nuances such as air drafts, uneven heating, and small leaks in tubing. In short, the VAN procedure is a time consuming process, which must be carefully monitored.

Since acetic acid is the major acid being measured by the VAN analysis, it is feasible that assessment of the acetic acid content of a fish extract by means of a commercially available enzyme kit would be quicker and more specific, and would lend itself to the processing of large numbers of samples. The objective of the present research was to compare the VAN procedure for measuring volatile organic acids, predominately acetic acid, with a method for measuring acetic acid enzymatically. The first part of this research concentrated on measuring acetic acid enzymatically in various types of fish and comparing the results with those obtained from the VAN procedure. In addition, TVB and TBA were used to further evaluate seafood quality. During the second part of the study, decomposition studies were performed on a fatty fish (mackerel) and on a lean fish (cod) to determine the usefulness of various chemical parameters for assessing seafood quality, and to compare the results from the VAN procedure with the values from the enzymatic acetic acid method.

Experimental

Sample Preparation

Fresh and canned fish.—Various types of fresh fish and canned fish packed in water or oil were obtained from local distributors and markets. Fresh fish was stored in a refrigerator maintained at 0–4°C. On the day of analysis, the fish was finely chopped and thoroughly mixed. Portions of the fish sample were weighed for the following analyses: total volatile bases, volatile acid number, and acetic acid determination by means of enzyme method and by titration with 0.01N NaOH. The thiobarbituric acid method for measuring malonaldehyde was performed when sufficient sample was available.

Decomposition Studies

Fresh degutted cod (*Gadus morhua*) and mackerel (*Scomber scombrus*) in iced condition were obtained from local distributors and transported on ice to the laboratory

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Table 1. Recovery statistics of various methods used to measure fish decomposition

Statistic	TVB[a]	TBA[b]	VAN[c]	Acetic acid[d]
Trials	24	9	24	23
Mean recovery, %	95.0	70.4	54.0	103.2
Std dev.	2.9	2.4	2.9	6.0
Range, %	10.0	5.8	10.9	20.4

[a] Total volatile bases (mg N/100 g).
[b] Thiobarbituric acid (μg malonaldehyde/g sample).
[c] Volatile acid number.
[d] Acetic acid enzymatic (mg/100 g).

where fillets were immediately prepared and the skin was removed. To ensure that the samples tested would be as uniform as possible in tissue composition and that introduction of other contaminants by handling would be minimized, the fillets from each type of fish were coarsely chopped and mixed manually before being divided into individual portions. Eight to 10 portions, in 100 g amounts, of chopped and mixed fish tissue were weighed into 250 mL beakers, covered with paraffin, and stored at 0–4°C until the day of analysis.

Blending of the fish was avoided for 2 reasons; Hultin et al. (9) note that lipid oxidation may become a problem in fatty fish that are thoroughly minced, and preliminary studies in this laboratory indicated that blending combined with the addition of 20% phosphotungstic acid (for protein precipitation) formed an emulsion which, after filtering, did not give a clear filtrate.

One beaker of tissue for each type of fish was analyzed immediately to determine baseline values. At various intervals, the separated test portions of tissue were analyzed for the following: TVB, TBA, VAN, titrimetric acetic acid, and enzymatic acetic acid.

Methods of Analysis

Total volatile bases (TVB).—Test was performed to measure the extent of proteolytic degradation and formation of other nitrogenous compounds such as trimethylamines. Test was conducted using the distillation procedure of Pearson (10).

Thiobarbituric acid test (TBA).—Test was performed on portions of tissue to determine the extent of lipid oxidation. Thiobarbituric acid test was performed by the distillation procedure of Tarladgis et al. (11) with the following modification: 10 g minced fish tissue was weighed and placed in a Kjeldahl flask followed by addition of 10 g NaCl, 98 mL water, and 2 mL HCl (1 part concentrated HCl to 2 parts of water). The method of Holland (12) was followed in setting up the standard curve.

Volatile acid number (VAN).—Test was conducted according to the standard procedure described in sections **18.050, 18.052–18.054** (5).

Enzymatic acetic acid.—Content was determined using an acetic acid test kit distributed by Boehringer-Mannheim Biochemicals (Cat. No. 148261, Indianapolis, IN 46280). Sample analysis was performed per instructions with the exception of the sample preparation. A 20 mL portion of filtrate from the VAN extraction procedure was used for determination of enzymatic acetic acid. The filtrate was neutralized with NaOH (5N to 0.01N) to ca pH 7.5–8.0, and diluted to 25 mL total volume with water; this gave a dilution factor of 1.25 and a total dilution factor of 7.5 (1.25 × 6 which represented the dilution factor resulting from original sample of 50 g in a total volume of 300 mL). In those cases where an extra 20 mL filtrate was not available after transfer of 150 mL for VAN analysis, the same dilution factor was retained by using 8 mL filtrate, neutralizing, and diluting to 10 mL total volume.

Table 2. Summation of raw data for measures of quality for various fresh fish species

Sample	Seafood	TVB,[a] mg N/100 g	VAN[b]	AA[c] titrimetric, mg/100 g	AA[c] enzymatic, mg/100 g
1	New Zealand roughy	11.9	0.0	0.0	0.0
2	swordfish	15.1	2.0	2.2	0.0
3	pollock	13.6	2.8	3.2	2.0
4	Atlantic smelt	13.7	3.2	3.6	1.1
5	cod	23.5	4.2	4.7	2.1
6	cod	26.9	4.4	5.0	0.0
7	haddock	19.2	4.5	5.0	2.2
8	perch	13.3	5.2	5.9	6.9
9	smelt	25.5	5.6	6.2	0.0
10	haddock	23.4	5.6	6.2	0.0
11	cod	30.8	5.9	6.6	3.9
12	salmon steak	28.7	6.4	7.1	8.9
13	sole	17.2	6.8	7.5	4.7
14	whiting	31.8	6.8	7.5	0.0
15	swordfish	23.8	7.2	8.0	0.0
16	whiting	30.4	8.7	9.6	2.2
17	swordfish	17.8	9.1	10.0	3.6
18	salmon	23.1	9.6	10.7	12.5
19	ocean spot	26.3	14.0	15.6	9.0
20	flounder	24.5	16.8	18.6	15.8
21	mackerel	46.6	20.3	22.5	16.2
22	cod	33.9	23.2	25.8	20.5
23	haddock	29.8	25.2	28.0	28.1
24	whiting	58.1	31.2	34.7	36.9
25	scrod	32.9	34.4	38.3	34.8
26	fluke	52.8	47.6	53.0	60.6
27	scrod	78.4	48.4	53.9	52.6
28	redfish	70.7	52.8	58.7	37.9
29	catfish	73.1	62.4	69.3	46.4
30	bluefish	46.9	65.2	72.5	54.3
31	smelt	60.6	68.0	75.6	60.0
32	herring kipper	25.9	84.8	94.2	62.3
33	haddock	75.3	102.0	113.4	116.2
34	cod	82.6	106.8	118.7	127.5
35	cod	79.4	115.2	128.0	114.5
36	mackerel	49.7	116.0	128.9	113.4
37	pollock	74.9	118.0	131.1	113.3
38	cod	112.4	190.0	211.0	206.3

[a] Total volatile bases.
[b] Volatile acid number.
[c] Acetic acid.

Titrimetric acetic acid.—Value was calculated from the VAN number by using titrated amount of 0.01N NaOH as follows:

$$\text{Acetic acid, mg/100 g tissue} = (\text{mL 0.01N NaOH titrated/A}) \times (B \times C \times D \times E)$$

where A = average percent recovery = 54% (0.54); B = normality of NaOH = 0.01N; C = g equiv. weight of acetic acid = 60.05; D = factor to convert mg/50 g tissue to mg/100 g tissue = 2; E = dilution factor (150 mL filtrate from original 300 mL) = 2.

Quality Assurance and Standard Recovery

Blanks, replicates, and percent standard recoveries were included each time a sample or samples were assayed. Standard recoveries were performed by adding a known amount

Table 3. Summation of raw data for measures of quality for various canned fish

Sample	Seafood	TVB,[a] mg N/100 g	TBA,[b] μg/g	VAN[c]	AA[d] titrimetric, mg/100 g	AA[d] enzymatic, mg/100 g
1	sardines (oil)	54.6	0.53	6.0	6.6	3.2
2	sea kisses (water)	8.8	nd[e]	7.2	8.0	4.8
3	sardines (oil)	46.2	2.12	7.6	8.4	4.5
4	tuna (oil)	41.7	0.39	9.2	10.2	6.3
5	anchovies (oil)	nd	nd	9.2	10.2	6.4
6	tuna (water)	40.8	0.25	9.6	10.7	5.2
7	anchovies (oil)	nd	nd	9.6	10.7	4.8
8	anchovies (oil)	nd	nd	10.0	11.1	7.7
9	tuna (water)	48.0	0.13	10.8	12.0	7.5
10	mackerel (oil)	45.2	1.12	10.8	12.0	14.8
11	mackerel (oil)	39.9	0.41	12.0	13.4	6.6
12	mackerel (oil)	41.7	1.76	12.0	13.4	7.3
13	mackerel (oil)	41.3	0.59	12.0	13.4	6.3
14	tuna (water)	38.3	0.17	12.4	13.8	5.9
15	mackerel (oil)	44.1	0.41	13.2	14.7	8.0
16	sardines (oil)	50.2	nd	15.2	17.0	9.5
17	fish steaks (oil)	53.9	2.04	16.0	17.9	12.8
18	sardines (tomato sauce)	41.3	0.66	20.0	22.2	11.6
19	mackerel (oil)	39.9	0.50	20.4	22.7	13.9
20	kipper (oil)	nd	nd	22.9	25.5	18.1
21[f]	crab (oil)	16.1	0.25	35.0	38.9	1.1
22	tuna (water)	nd	nd	78.0	86.7	66.5
23	tuna (water)	32.2	0.48	114.0	126.6	144.7

[a] Total volatile bases.
[b] Thiobarbituric acid for malonaldehyde.
[c] Volatile acid number.
[d] Acetic acid.
[e] No data available due to insufficient sample quantity.
[f] Sample is an outlier according to Grubbs and Dixon tests and was not included in further statistical tests.

of analyte in place of the sample and running the standard concurrently with samples. The following standards were used: for TVB—1 mL 0.1N NH_4Cl = 1.4 mg N; for TBA—10 mL of a solution of 4 μg malonaldehyde/mL as prepared from 1,1,3,3-tetraethoxypropane (TEP), where 0.306 g TEP = 1.00 mg malonaldehyde (No. T9889, Sigma Chemical, St. Louis, MO 63178); VAN—50 mL 0.1N acetic acid = 5 meq acetic acid; enzymatic acetic acid—10 μg per cuvet (0.1 g/L).

Results and Discussion

Table 2 shows the recovery statistics for the various chemical parameters used in this study for the assessment of seafood quality. Mean standard recoveries for TBA and VAN methods were low, but these values are in line with published recoveries. Tarladgis et al. (11) report 66–70% recovery for malonaldehyde in 50 mL TBA distillate; Clark and Hillig (13) report a mean recovery of 57.2% for the VAN method.

The results of the various chemical tests performed on raw

Table 4. Regression equations, correlation coefficients (r), and coefficients of determination (r^2) comparing acetic acid test methods with volatile acid number and TVB

Test comparison (X, Y)	N[a]	Regression equation	r	r^2
		Fresh fish		
VAN,[b] enzymatic AA[c]	38	Y = 1.05X − 3.59	0.986	0.973*
Titrimetric AA,[d] enzymatic AA[c]	38	Y = 0.95X − 3.60	0.986	0.973*
TVB,[e] VAN	38	Y = 1.56X − 24.55	0.861	0.742*
TVB,[e] enzymatic AA[c]	38	Y = 1.67X − 30.80	0.868	0.746*
TVB,[e] titrimetric AA[d]	38	Y = 1.73X − 27.26	0.861	0.742*
		Canned fish		
VAN,[b] enzymatic AA[c]	22	Y = 1.20X − 6.75	0.980	0.960*
Titrimetric AA,[d] enzymatic AA[c]	22	Y = 1.08X − 6.78	0.980	0.960*
TVB,[e] VAN[b]	22	no linearity	−0.210	0.044
TVB,[e] enzymatic AA[c]	22	no linearity	−0.216	0.047
TVB, titrimetric AA[d]	22	no linearity	−0.209	0.044

[a] Number of samples assayed.
[b] Volatile acid number.
[c] Acetic acid (mg/100 g) determined enzymatically.
[d] Acetic acid (mg/100 g) determined titrimetrically.
[e] Total volatile bases (mg/100 g).
* $P < 0.01$.

Table 5. Results of paired *t*-tests comparing VAN and acetic acid methods

Test comparison	N[a]	Calcd *t*-value	Critical *t*-value[b]
Fresh fish			
VAN[c] vs enzymatic AA[d]	38	1.240*	2.026
Titrimetric AA[e] vs enzymatic AA[d]	38	4.300**	2.026
Canned fish			
VAN[b] vs enzymatic AA[d]	22	1.642*	2.080
Titrimetric AA[e] vs enzymatic AA[d]	22	3.575**	2.080

[a] Number of pairs analyzed.
[b] Critical Students's *t*-value at 0.05 level of significance.
[c] Volatile acid number.
[d] Acetic acid (mg/100 g) measured enzymatically.
[e] Acetic acid (mg/100 g) measured titrimetrically.
* No significant difference between means.
** Significant difference between means.

and canned fish, sorted from lowest to highest according to VAN values, are presented in Tables 2 and 3. In general, VAN measurements correlated well with acetic acid values for both the fresh fish and canned fish. Earlier studies on cod and flounder (1, 6) report a similar association between VAN and acetic acid values, although the method used for assessing acetic acid in these studies was not an enzymatic procedure.

Although TVB values for the fresh fish were higher than corresponding VAN and acetic acid values, low TVB measurements were linked with low VAN and acetic acid values. Differences between TVB, and VAN and acetic acid values are most likely the result of measuring different substances. Total volatile bases is a measure of nitrogenous protein decomposition and nonprotein nitrogenous decomposition (notably trimethylamine from trimethylamine oxide), whereas VAN and acetic acid procedures primarily assess acetic acid.

In contrast to the fresh fish, the canned fish showed no relationship between TVB values and VAN or acetic acid measurements. TVB values for the canned fish remained fairly constant, with most of the measurements falling between 40 and 50 mg TVB-N/100 g. Hillig et al. (1) comment that basic nitrogen compounds are unsatisfactory for the detection of decomposition in canned fish products since some of these products are liberated in processing.

Results of the linear regression analyses performed on the raw data for fresh and canned fish using the least-squares method (14) are presented in Table 4. Coefficients of determination for volatile acid number and titrimetric acetic acid vs enzymatic acetic acid were greater than or equal to 0.96 for both fresh and canned fish, indicating significant positive correlations. Corresponding high correlation coefficients were also observed among these same parameters. The slopes of the regression lines for VAN vs the enzymatic acetic acid were close to 1, indicating an almost 1:1 correlation between these values. Although significant positive correlations for TVB vs VAN and the acetic acid methods were noted in the fresh fish (r^2 = 0.74, 0.75, 0.74 respectively), these correlations were lower than the correlations observed between VAN and titrimetric acetic acid vs the enzymatic acetic acid ($r^2 > 0.97$). There were no significant linear correlations between TVB vs VAN and acetic acid methods in the canned fish.

Results of Student's paired *t*-test are presented in Table 5. There was no significant difference between VAN and enzymatic acetic acid results for fresh and canned fish. The reason for the significant difference seen between titrimetric

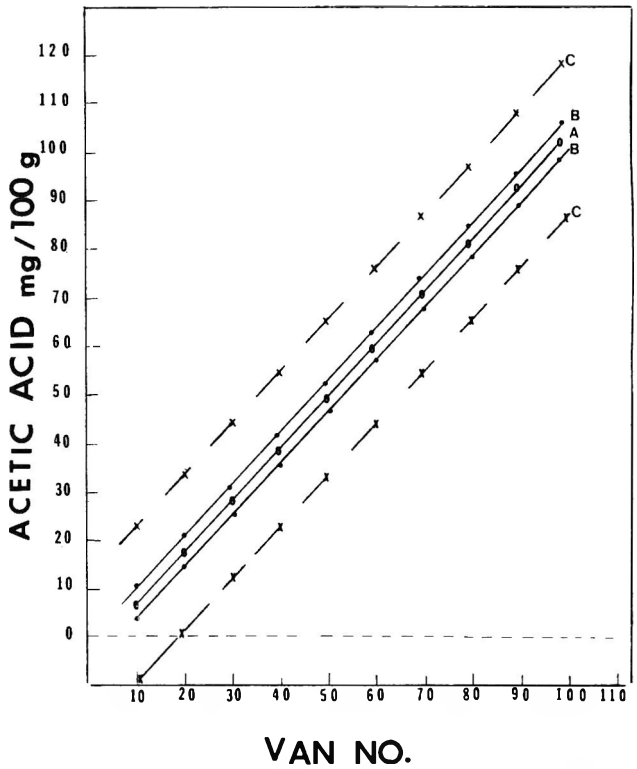

Figure 1. Predicted values of enzymatic acetic acid from VAN: A, regression line of enzymatic acetic acid (Y) on VAN (X); B, 95% confidence limits to include true Y; C, 95% confidence limits on an individual Y.

acetic acid and enzymatic acetic acid in both fresh fish and canned fish is speculative, but probably can be attributed to the different types of fish sampled and the variation in percent recovery used in calculating the titrimetric acetic acid.

Figure 1 shows the 95% confidence limits for the regression line and single point observation for prediction of enzymatic acetic acid from VAN. The upper and lower confidence limits for 10 VAN values, ranging from 10 to 100, averaged 3.05 mg/100 g for the regression line and 16.12 mg/100 g for an individual Y value.

The chemical changes that occurred during storage of a lean fish (cod) and a a fatty fish (mackerel) at 0–4°C are shown in Figures 2 and 3. Total volatile bases, VAN, acetic acid, and TBA were measured until the fish were noticeably (as determined by off odor and slimy texture) in an advanced stage of decomposition. Acetic acid and VAN values paralleled each other closely for both the lean fish and fatty fish. Volatile acid number and acetic acid values began to increase sharply around day 2 for both types of fish. The 2 indices continued rising until day 4 in the cod and day 6 in the mackerel, followed by a more gradual increase for both. Between day 8 and day 9, VAN and titrimetric acetic acid values took another sharp rise in the fatty fish. This sharp increase in volatile acids may be due to acids other than acetic being generated and to fatty acid oxidation. By day 8, the mackerel was organoleptically evaluated to be in an advanced stage of decomposition. TVB values followed a similar pattern to VAN and acetic acid parameters in the lean fish over the entire study. In contrast, TVB values in the fatty fish showed a more gradual increase. Results of TBA determination are diametrically opposed in the 2 types of fish; there was little variation in these values for cod throughout the study, but values for mackerel increased sharply between day 2 and day 3 and then fluctuated with rapid increases and decreases. Some of these changes may be due to sample

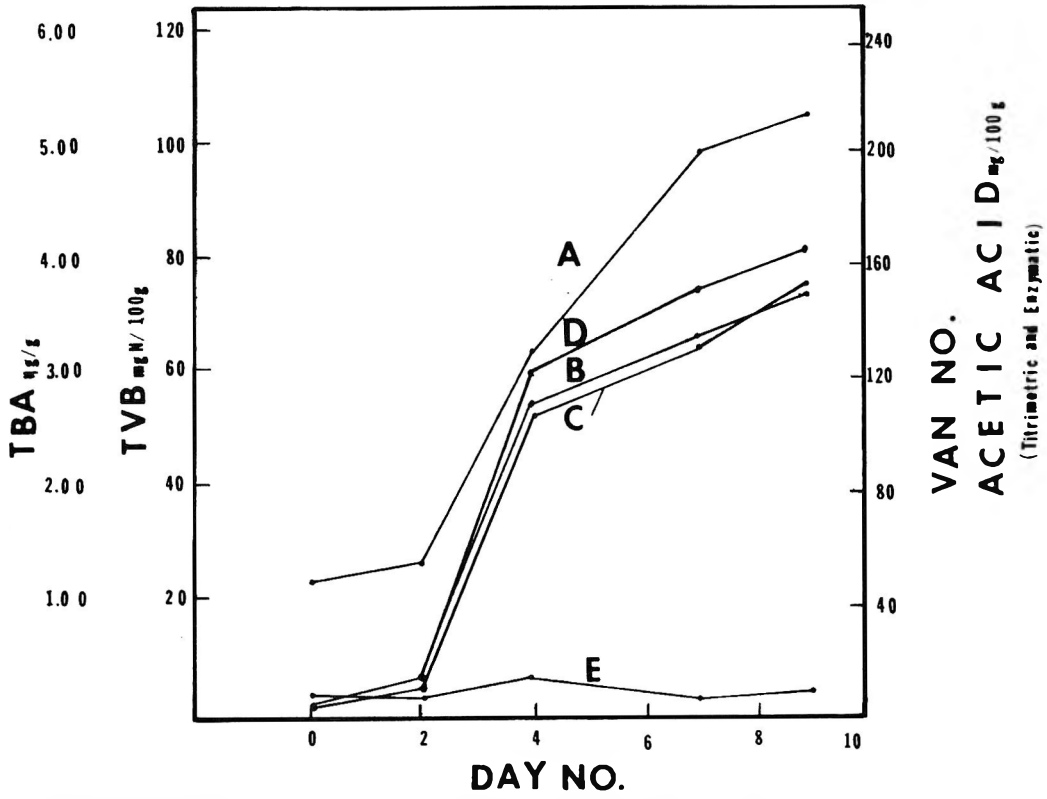

Figure 2. Changes in TVB (A), VAN (B), enzymatic acetic acid (C), titrimetric acetic acid (D), and TBA values for malonaldehyde (E), during storage of cod fillets at 0–4°C.

variation, but Wood and Hintz (15) note similar increases in TBA during the first 6 days with a decrease thereafter. They suggest that the decrease may reflect the usefulness of the test in the early stages of oxidation.

In this study, the point at which the fish exhibited organoleptic signs of decomposition (off odor, sliminess) was different for lean and fatty fish. Mackerel, which is considered a fatty fish (12.2% approximate fat composition compared to 0.3% for cod (16)), began to show a decrease in freshness (slight off odor) around day 3 and was listed unsatisfactory by day 6. This is consistent with the results of Jhaveri et al. (17) who report that Atlantic mackerel that is stored on ice remains fresh, as judged by organoleptic criteria, for 0 to 6 days. The time between day 3 and day 6 is also the time when the VAN and acetic acid methods are most closely correlated, and when TBA shows the greatest increase before beginning to decline. This indicates that TBA in combination with VAN and/or enzymatic acetic acid is an appropriate marker for fatty fish assessment. Based on the decomposition study, a VAN value of about 40 would indicate a beginning phase of spoilage for mackerel. This value would correspond to 38 mg/100 g of acetic acid as determined by the regression equation for VAN vs enzymatic acetic acid for fresh fish (Table 4).

Cod began to show organoleptic signs of spoilage on day 4 and was listed as unsatisfactory by day 9. The greatest increases in the chemical parameters occurred between days 2 and 5. These increases are similar to the results of Sullivan et al. (18), who observed the greatest increases in the chemical parameters between days 2 and 4 for flounder stored at 5°C. After 4 days of storage, the flounder was considered spoiled by organoleptic criteria.

In the present study, the results (Figure 2) showed that, although the chemical and organoleptic measures of spoilage overlapped, the organoleptic indication of spoilage lagged behind the chemical markers. The average TVB value on day 4, which is the first day that cod showed some signs of spoilage (odor), was 64 mg TVB-N/100 g. Varga et al. (19), who used an experienced test panel and chemical analyses to assess freshness of cod fillets, show fresh fish to have TVB-N of ca 20 mg/100 g, marginal fish ca 60 mg TVB-N/100 g, and inedible fish > 80 mg TVB-N/100 g. Cox and Pearson (20) suggest 30 mg TVB-N/100 g to be the limit of freshness, but they also note that this value may not coincide with organoleptic measures of spoiled fish. Sullivan et al. (18) also comment that several samples that had more than 60 mg TVB-N/100 g displayed satisfactory appearance and odor.

Based on in-laboratory studies and taking into consideration these suggested values and comments, 40 mg TVB-N/100 g might serve as a reasonable cutoff value for acceptability in lean fish when combined with an organoleptic evaluation and further verification using the volatile acid number method. According to Figure 2, a value of 40 mg TVB-N/100 g would correspond to a VAN value of about 45 and an enzymatic acetic acid of about 38 mg/100 g. The enzymatic acetic acid value calculated from the VAN value of 45, using the regression equation for fresh fish (Table 4), resulted in an acetic acid value of 44.4 mg/100 g. Both the VAN and enzymatic acetic acid values determined from the time study, and the enzymatic acetic acid value calculated from the regression equation, are in agreement with a VAN value of 39 which Hillig et al. (1) report as the maximum value for a Class 1 or acceptable quality codfish.

In addition, correlation coefficients were determined for VAN vs enzymatic acetic acid for samples of fish grouped according to acceptable and unacceptable quality, using a VAN of 40 as the cutoff value for acceptability. Correlations of 0.93 and 0.96 were calculated for the acceptable and

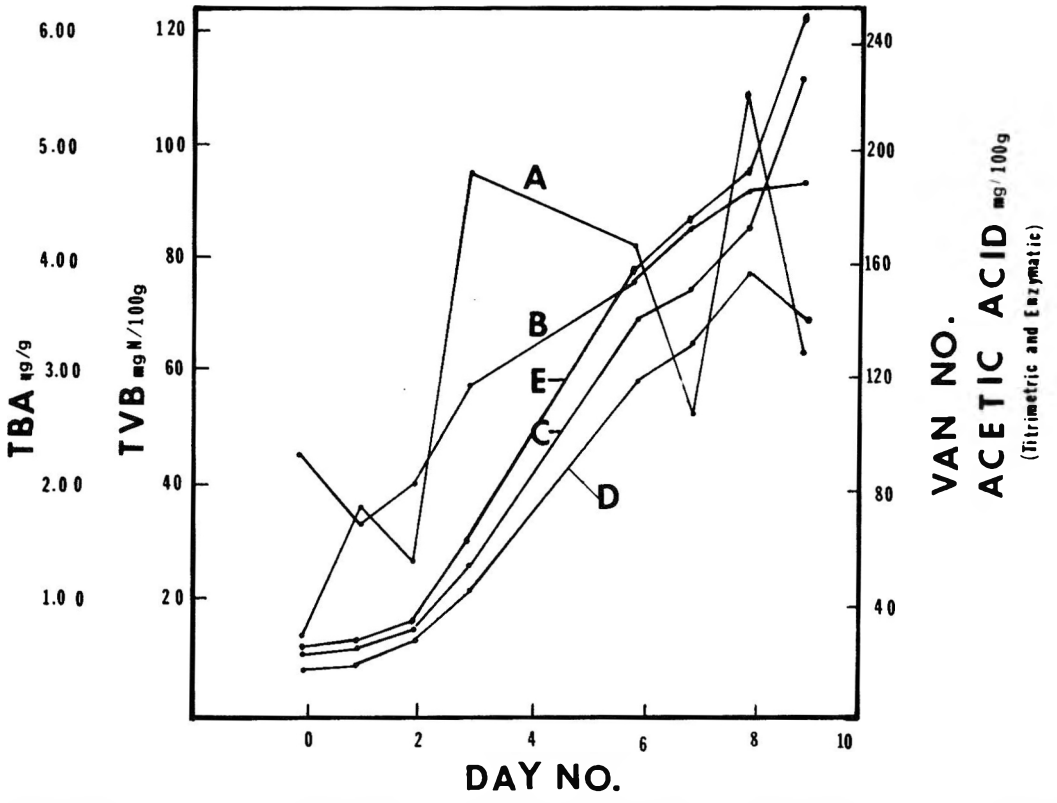

Figure 3. Changes in TBA values for malonaldehyde (A), TVB (B), VAN (C), enzymatic acetic acid (D), and titrimetric acetic acid (E), during storage of mackerel fillets at 0–4°C.

unacceptable group, respectively. Thus, under the conditions of this analysis, the enzymatic acetic acid values correlate well with the VAN values at both the high and low levels.

The high correlations between VAN and acetic acid values that were observed in this study suggest that the measurement of acetic acid by the enzymatic method is comparable to that of the VAN method. Recognizing that a reliable evaluation of a particular sample of seafood involves assessment using several different chemical parameters in combination with an organoleptic evaluation, the results of this study suggest that enzymatic acetic acid can be a reliable indicator of the quality of certain seafood when used in combination with other chemical parameters such as TVB for lean fish and TBA for fatty fish.

Enzymatic acetic acid as one indicator of seafood quality has certain advantages over the AOAC volatile acid number method. The enzyme kit allows for the evaluation of a larger number of samples in a shorter time. While it takes approximately 1 day to process 7 or 8 samples using the VAN method, 25 to 30 samples can easily be processed in a day using the enzyme kit. The distillation apparatus necessary for the VAN method is cumbersome and sensitive to environmental conditions whereas the enzymatic acetic acid procedure requires little equipment, other than a spectrophotometer capable of recording absorption at 340 nm, and produces results that are consistent from day to day. Although the principal component in the 200 mL distillate collected for the VAN procedure is acetic acid (1–3), other volatile acids may also distill over and be included in the final value. The enzymatic acetic acid method by its very nature is specific for acetic acid.

In conclusion, volatile acid number and enzymatic acetic acid are comparable markers for assessing seafood quality, but the enzymatic procedure offers certain advantages for those laboratories needing to routinely process large numbers of samples.

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WATER

Liquid Chromatographic Determination of Total Formaldehyde in Drinking Water

BRUCE A. TOMKINS, JOHN M. McMAHON, AND WILLIAM M. CALDWELL
Oak Ridge National Laboratory, Organic Chemistry Section, Analytical Chemistry Division, PO Box 2008, Oak Ridge, TN 37831-6120
DAVID L. WILSON
Oak Ridge National Laboratory, Health and Safety Research Division, PO Box 2008, Oak Ridge, TN 37831-6113

Formaldehyde is readily quantitated at μg/L levels in drinking water. The analyte present in 1 L water samples is derivatized with 2,4-dinitrophenylhydrazine in a 2M acid medium and then extracted with chloroform. After the solvent is exchanged for methanol, the product is separated and quantitated using reverse-phase liquid chromatography with UV detection (365 nm). Reporting limits as low as 20 μg/L (corrected for laboratory blank) are routinely achieved. Formaldehyde recovery typically exceeds 90% at 20–200 μg/L. The method was applied to hot and cold water samples from thirty-four 1- and 2-story houses equipped with poly(acetal) plumbing elbows and tees. The drinking water samples were accompanied by sets of blanks and spikes specified by a quality assurance/quality control plan. Formaldehyde was observed above the reporting limit in 80% of hot and cold water samples from the 1-story dwellings, but in less than 50% of those from the 2-story dwellings. The results may depend on both the construction of the houses (1- vs 2-story) and the time of year the water is sampled (mid-summer).

Formaldehyde, an animal carcinogen, causes squamous cell carcinomas in the nasal cavities of laboratory rats and male mice (1, 2). Graftstrom et al. (3) reported that this compound caused the formation of undesirable cross-links between DNA and proteins, caused single-strand breaks in DNA, and inhibited the resealing of single-strand breaks produced by ionizing radiation. Those authors concluded that formaldehyde can exert its mutagenic and carcinogenic effects by both damaging DNA and inhibiting its repair (3). These data were sufficient for the U.S. Environmental Protection Agency (EPA) to designate formaldehyde for priority review under the Toxic Substance Control Act (4).

Because of the difficulty of analysis and sparsity of data, EPA has not established a regulatory standard for formaldehyde in drinking water. The Emergency Response Group Committee on Toxicology, National Academy of Sciences, has recommended that EPA establish a drinking water standard of 110 μg/L. In contrast, California and New Jersey have enforceable action levels of 30 and 0.65 μg/L, respectively, based on 2 very different and independently derived physiological models (5, 6).

Plastic plumbing fixtures (e.g., elbows and tees) fashioned from poly(acetal) can be hydrolyzed by either hot or cold water (personal communication, 1985, J. C. Heim, Brown & Caldwell Analytical Laboratories, Emeryville, CA). The resulting formaldehyde, which leaches slowly and continuously from these pieces and, thereby, enters the drinking water, may exceed suggested regulatory standards.

Clearly, there is a pressing need to develop, test, and validate a practical analytical method for quantitating formaldehyde in drinking water at μg/L levels. Direct-injection gas chromatography with either flame or helium ionization detectors does not exhibit sufficient sensitivity to be useful (7). The latter can detect formaldehyde at concentrations of less than 1 mg/L; however, routine quantitation of formaldehyde at the 3 regulatory levels described would not be possible.

At least 2 authors (8, 9) have attempted to concentrate derivatized or underivatized formaldehyde on solid sorbents. Ogawa and Fritz (8) found that small columns packed with zeolite ZSM-5 concentrated μg/L concentrations of low molecular weight aldehydes and ketones from aqueous samples; however, this procedure exhibited very poor (about 1%) recoveries for formaldehyde at 100 μg/L. Takami et al. (9) demonstrated that moderately sulfated cation exchange resin cartridges charged with 2,4-dinitrophenylhydrazine could indeed concentrate formaldehyde present at μg/L levels (1–10 μg/L) from drinking water. However, we were not able to reproduce these results in our laboratory using either prepacked silica-based sulfonic acid cartridges with 0.45 meq/cartridge ion exchange capacity (as specified in ref. 8) or polystyrene resin-based cartridges (packed by hand) with 10 times the ion exchange capacity.

An alternative approach is to quantitate formaldehyde as a derivatized product by conventional liquid-liquid extraction and concentration procedures. The literature suggests that the reagents 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (10–13); dansyl hydrazine (14); and 2,4-dinitrophenylhydrazine (DNPH) (15) might be appropriate for UV detection. Dong and Dasgupta (16) described a fast fluorometric flow injection procedure for quantitating formaldehyde with 2,4-pentanedione and ammonium acetate as the reagents. Their method exhibits μg/L sensitivity for formaldehyde, but it was tested on fogwater, rather than drinking water, where the formaldehyde levels are in the mg/L, not μg/L, range.

We adapted that method (15) to quantitate formaldehyde in drinking water for 2 reasons. (*1*) A literature procedure exists (15) that demonstrates DNPH will rapidly and quantitatively derivatize many small aldehydes and ketones (including formaldehyde) present in the vapor-phase of cigarette smoke. (*2*) The derivative of formaldehyde is readily separated from other potentially interfering species by liquid chromatography. Our procedure exhibits good sensitivity (routine reporting limit about 20 μg/L), adequate resolution of the formaldehyde derivative from other potential interferences, and reasonable speed and ease of manipulation. Our method was rigorously tested using a variety of blanks and spiked samples containing formaldehyde at low μg/L concentrations.

Bicking et al. (17, 18) suggested that any analyses of formaldehyde in water with 2,4-dinitrophenylhydrazine as the reagent should be performed with the mildest possible level of acid catalyst. This precaution would minimize additional formaldehyde formation because of acid hydrolysis of certain matrix precursors, and would, thus, yield a measurement of "free" formaldehyde. Our method, which uses strongly acidic conditions and, thereby, promotes such hy-

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drolysis, yields a measurement of "total" formaldehyde. Such a value would estimate the maximum formaldehyde hazard of a given water sample.

METHOD

Apparatus

(a) *Liquid chromatography.*—Hewlett-Packard Model 1090, or equivalent, with diode array detector, computer-console control, syringe-type injector (1–25 μL range), and Think-Jet® printer. Use integration and reporting capabilities of instrument for quantitation.

(b) *Sample bottles.*—1 L narrow-neck amber I-Chem (Hayward, CA) amber glass with Teflon-lined closures attached.

Reagents

(a) *Solvents.*—Chloroform, methanol, acetonitrile, and water, distilled in glass (Burdick & Jackson Laboratories, Muskegon, MI).

(b) *Hydrochloric acid (HCl, 0.100N, 1N, 2N, and 12N).*—Reagent grade, use as received. Determine exact concentration of 0.100N HCl using titration procedure.

(c) *2,4-Dinitrophenylhydrazine (DNPH).*—Aldrich Chemical Co. (Milwaukee, WI), Eastman Kodak Co. (Rochester, NY), or J. T. Baker Co. (Phillipsburg, NJ). *Caution:* This reagent is explosive when dry, and is normally supplied with at least 10–30% w/w water. Do not dry the reagent before use.

(d) *DNPH reagent solution.*—Saturate 1 L 2N HCl with DNPH (solubility ca 300 mg/L).

(e) *Formaldehyde stock solution (ca 4 mg/mL).*—Purchase reagent grade formaldehyde, 37% solution, from EM Science (Cherry Hill, NJ), or equivalent. Pipet 5.00 mL into 500 mL volumetric flask and dilute to volume with distilled, deionized water. Warm solution to ca 30°C, and cool to room temperature to completely dissolve formalin. This solution is ca 4 mg/mL in formaldehyde. Determine exact concentration using titration procedure described below.

(f) *Sodium sulfite solution (1.0M).*—Dissolve 63.02 g reagent grade sodium sulfite in 500 mL distilled, deionized water.

(g) *Indicator solution.*—Dissolve 40 mg indicator grade thymolphthalein in 40 mL reagent grade ethanol.

(h) *Standard solutions.*—Pipet 625 μL formaldehyde stock solution into 100 mL 2N HCl saturated with DNPH. Extract twice with 50 mL portions of chloroform and pool layers. Wash chloroform twice with 50 mL portions of 1N HCl and once with 100 mL water. Remove chloroform by rotary evaporation; do not let residue become completely dry. Redilute residue to exactly 50 mL in methanol. Resulting 50 mg/L derivatized formaldehyde stock is stable at least 6 months when refrigerated at 4°C. Dilute 50 mg/L stock to 1, 2, 5, and 10 mg/L derivatized formaldehyde in methanol. These diluted standards are also stable at least 6 months when refrigerated at 4°C.

Drinking Water Samples

Water was drawn from twelve 1-story and twenty-two 2-story houses and collected in separate sample bottles. Water (25 mL) collected first in a graduated cylinder was discarded to avoid contamination from soap, rubber gaskets in faucets, etc. All sample bottles were filled to overflowing to eliminate headspace. Cold water was sampled first then hot water. Indoor sampling was performed at least 8 h after water supply was last used. Tap water aerators, filters, or other devices were removed from faucets before collection. If sample could not be taken from sink faucet, it was collected from bathtub faucet.

Using same procedure, additional water samples were collected from outside spigots at every third residence (a total of 11 samples). These outside aliquots were used as field blanks because this water carried in all-copper lines had not yet come into contact with poly(acetal) fittings inside houses.

All drinking water samples were preserved at the end of each day using method described below.

Quality Assurance/Quality Control

Because extraction is both physically demanding and time-consuming, some samples were stored for several weeks before processing. To ensure proper quality control, experimental controls were devised. Two empty sample bottles from one lot were chosen at random and filled with distilled-in-glass water and preservative reagents described below. Bottles were then allowed to "age" for approximately 2 weeks before extraction.

Field blanks were collected at every third house from all-copper outside spigots. They were then distributed evenly throughout the analysis schedule so that each set of samples extracted would contain at least 1 field blank. These blanks were used to establish background formaldehyde level.

Ten laboratory storage blanks were collected from single spigot outside a house with no known poly(acetal) fittings. Two of these samples were analyzed each week during course of analysis. These samples were included to identify any contamination problems associated with storage or extraction.

Trip blanks of distilled-in-glass water were generated on-site to confirm that no contamination of the samples occurred during transport.

At time of sample collection, 2 identical sets of spiked samples at 0, 20, 30, and 60 μg/L of formaldehyde were prepared on-site with water samples drawn from outside spigots and freshly standardized 4 mg/mL aqueous formaldehyde stock solution. First set was analyzed before analysis of house samples to verify accuracy. Second set was analyzed after analysis of the house samples to determine chemical stability during storage.

Five additional spiked samples prepared at time of sample collection of three 60 μg/L, one 80 μg/L, and one 180 μg/L of formaldehyde, were analyzed at random points in the analysis schedule to measure accuracy of determination and stability of formaldehyde during course of analysis.

Finally, one pair of samples was collected inside house with no known poly(acetal) fittings. This extra set was to confirm that actual sampling technique was valid and that faucets did not contribute to formaldehyde concentrations. This information was, thus, complementary to laboratory storage blanks collected from all-copper outside spigots.

All blanks and spiked samples were preserved using method described below.

Standardization of Formaldehyde Stock Solution

Pipet 12.5 mL 1.0M sodium sulfite solution into 250 mL Erlenmeyer flask. Add 3 drops of thymolphthalein indicator. Resulting solution should be pale blue color. Add 0.100N HCl dropwise (usually requires 2–5 drops) until solution is colorless. By pipet, add exactly 25.00 mL formaldehyde stock to acidified sodium sulfite solution. Titrate dropwise to faint blue haze end point with 0.100N HCl. End point should persist 3 min. Perform 3 titrations and calculate mean value. Replicates should be within 0.3%. Calculate exact concentra-

tion of formaldehyde stock solution as follows:

$$\text{HCOH (mg/mL)} = [\text{HCl titrant (mL)} \times 0.1 \text{ mmol HCl/mL} \times 30.03 \text{ mg HCOH/mmol}]/25.00 \text{ mL}$$

Sample Preservation

Preserve all blanks, spikes, and drinking water samples by first removing 60 mL water and then adding 10 mL 12N HCl and 300 mg DNPH. Cap bottle and shake by hand for several minutes. Add 50 mL chloroform and pack bottle in cooler equipped with "Blue Ice" (0–4°C) for overnight shipment to laboratory for analysis. Store samples at 4°C.

Sample Preparation

Acidify each sample to 2N by adding 165 mL 12N HCl and then stir overnight at room temperature to dissolve remaining reagent. Extract each sample twice with 50 mL chloroform; pool layers. Wash chloroform layers twice with 50 mL 1N HCl and once with 100 mL water. Reduce volume of chloroform extract to near dryness by rotary evaporation. Dilute residue to exactly 25 mL with methanol.

Liquid Chromatography

Operating conditions: analytical column, Phenomenex Zorbax ODS, 7 μm particle size, 25 cm × 4.6 mm id (Rancho Palos Verdes, CA) equipped with Upchurch Scientific, Inc., dry-packed guard column (Perisorb RP-18, 30–40 μm) and precolumn coupler (Oak Harbor, WA). Mobile phase, methanol–water (70% v/v), flow rate 1 mL/min. Set wavelengths on diode array detector to 254 and 365 nm. Inject 25 μL sample onto LC column. Perform quantitation by electronic integration of peak area using 365 nm wavelength of diode array detector. Dinitrophenylhydrazone of formaldehyde (F-DNPH) typically elutes in ca 8 min under these conditions. Continue elution for additional 12 min to remove late eluting contaminants from column.

Calculations

Fit peak areas of F-DNPH at 1, 2, 5, and 10 mg/L to standard linear regression equation to obtain f(a), concentration of F-DNPH in mg/L as function of area (correlation coefficient typically exceeds 0.99). Calculate concentration of formaldehyde in μg/L for 1 L blank water sample, C(b), given by C(b) = 25 f(b), where (b) is peak area obtained from a blank extract. Average at least 5 independently determined blank values to estimate the method blank, M(b). Calculate concentration of formaldehyde in μg/L for a 1 L drinking water extract (uncorrected for blank), C(s) = 25 f(s), where (s) is peak area obtained from a single sample extract. Calculate concentration of formaldehyde corrected for blank, C(s) corr, as follows:

$$\text{C(s) corr, } \mu\text{g/L} = 25 \text{ f(s)} - \text{M(b)}.$$

Results and Discussion

The titration procedure described for standardizing the formaldehyde stock solution used in this procedure is reasonably straightforward. Some practice may be needed before the end point can be reproducibly detected. A correction is usually not necessary for the small quantity of 0.1N HCl added to render the solution colorless. The addition of up to 250 μL 0.1N HCl at the beginning of a trial will cause less than 1% error in a concentration of 4 mg formaldehyde/mL stock solution if a blank correction is not applied. For even greater accuracy, those initial few drops of 0.1N HCl may be added by buret and later subtracted as a blank.

The procedure described by Manning et al. was designed for the quantitation of small aldehydes and ketones in the vapor phase of cigarette smoke (15). The authors demonstrated that LC procedure was capable of separating derivatized peaks of small analytes, including formaldehyde, acetaldehyde, prcpionaldehyde, and acetone, with baseline resolution.

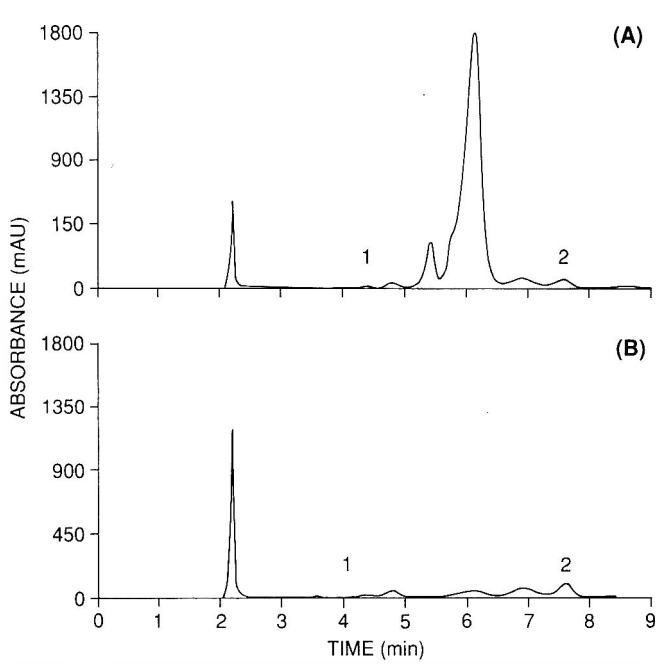

Figure 1. Typical chromatograms of a drinking water sample monitored at 254 nm (A) and 365 nm (B). Labeled peaks 1 and 2 are from reagent, 2,4-dinitrophenylhydrazine and 2,4-dinitrophenylhydrazone of formaldehyde, respectively. Concentration of formaldehyde present is 120 μg/L (corrected for blank).

The authors noticed that acetaldehyde eluted as 2 peaks that were not completely resolved. We observed a similar problem for formaldehyde when the derivative was injected in chloroform, but not when injected in methanol. We suspect that methanol is more miscible with the eluate than is chloroform, and the "double peaking" was caused by insufficient mixing. For this reason, chloroform was exchanged for methanol (about 95% is sufficient) before the LC analysis.

The 2,4-dinitrophenylhydrazone of formaldehyde, F-DNPH, absorbs strongly at both 254 and 365 nm. The latter wavelength was chosen for quantitative work for 3 reasons. First, the molar absorptivity of F-DNPH at 365 nm is twice that at 254 nm. Second, several species that interfered with the quantitation of F-DNPH absorbed much less strongly at 365 nm than at 254 nm. Finally, when chromatograms monitored at 254 nm were examined carefully, poor resolution was often observed between our analyte peak that eluted at about 8 min and an earlier eluting peak of equal or slightly greater peak area that eluted at about 7.2 min. This lack of resolution frequently resulted in a high bias for formaldehyde. The problem was not significant when 365 nm was used as the monitoring wavelength. These features are shown clearly in Figure 1. The eluant absorbance was also monitored at 254 nm to generate complementary information about each sample.

The procedure permits an operator to extract, concentrate, and chromatograph six 1 L water samples per working day (LC operates unattended overnight).

When a series of 8 laboratory blanks that contained no known formaldehyde was analyzed, a concentration of 40 ± 10 μg/L of formaldehyde in water was observed. The same

Table 1. Recoveries of formaldehyde from matrix spike samples

Calculated, μg/L	Measured, μg/L	Recovery, %
Analyzed Before Real Samples		
0	<20	—
20[a]	<20 (10)	50
30	32	107
60	63	105
Analyzed with Real Samples		
180	170	94
80	77	96
60	79	132
60	57	95
Analyzed After Real Samples		
0	<20	—
20	25	125
60	52	87

[a] Possible error in sample field preparation. Measured value is estimated.

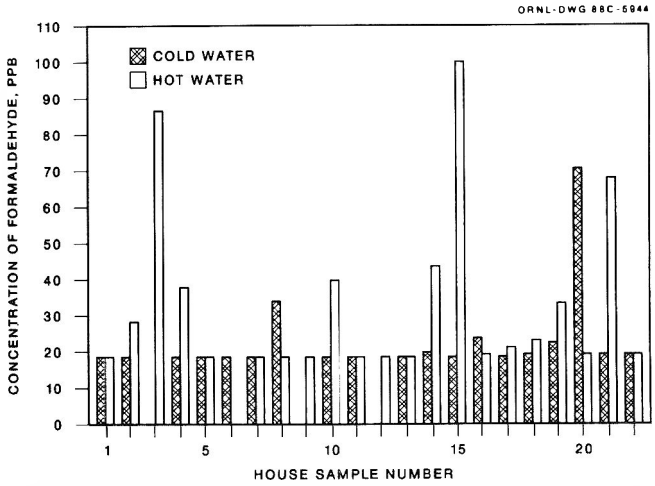

Figure 2. Formaldehyde concentrations in hot and cold water from 2-story homes. Shaded bars, cold water; empty bars, hot water.

observation was valid for a series of 11 field blanks, where drinking water had only passed through all-metal lines, had not contacted the poly(acetal) fittings, and had no known formaldehyde content. For this reason, the value of 40 μg/L was attributed to analytical method blank, rather than inherent contamination in either drinking or purified water (distilled-in-glass grade), and was, therefore, subtracted from all observed values. The cause for the laboratory blank value is not known; however, it is assumed to arise from interference unrelated to formaldehyde. Bicking et al. (17) reported a similar problem even when using much milder acid conditions than the present procedure. All data that appear in this paper have been corrected for the method blank.

The data system of the analytical instrument yielded a virtually noiseless baseline. Hence, calculation of the detection limit could not be based on typical parameters such as signal-to-noise ratio or the mean plus 3 standard deviations of the noise. As an alternative, the detection limit was based on the smallest measurable analyte peak. This value is about 3 μg/L of formaldehyde in drinking water.

The detection limit of formaldehyde was low enough to yield a significant number of false positives, particularly when data values were corrected for a method blank of 40 μg/L. For this reason, the reporting limit for formaldehyde was chosen to be half the method blank, to minimize the chance of false positives. The value chosen was 20 μg/L of formaldehyde in drinking water (corrected for blank), or about 7 times the detection limit.

Bottles can become contaminated with formaldehyde during cleaning unless strict rinsing procedures are obeyed. In one study, glass bottles were washed with tap water and a solution that contained phosphate-free detergent and a low level of formaldehyde as a preservative, followed by 3 rinses with distilled water and oven-drying. The resulting formaldehyde concentrations in those bottles were sufficient to render any data obtained completely useless. The bottles used in the present study were cleaned according to EPA protocols (acid and methylene chloride rinses only) by the manufacturer, and exhibited no residual formaldehyde. For that reason, additional cleaning was not required, and the bottles could be used "as received."

Because there were so many samples to be analyzed, our policy was to determine formaldehyde in groups that commonly contained hot and cold water samples, a trip blank, and 1 or more field blanks. This grouping served a 2-fold purpose. First, it was possible to have at least 1 blank, and often more, with each batch of drinking water samples. Second, it minimized possible loss of one type of sample data if the results were affected by short-term laboratory-based contamination problems. The latter occurred once during this study and caused the loss of 16 of 103 total samples.

In 7 of 8 laboratory storage blanks, the concentration of formaldehyde observed was less than 20 μg/L. The remaining sample exhibited 24 μg/L and was not deemed significantly different from the reporting limit. All 4 trip blanks exhibited formaldehyde concentrations less than 20 μg/L. Finally, 8 of the 11 field blanks showed concentrations of analyte less than 20 μg/L, 1 showed 20 μg/L (sample possibly contaminated due to a garden hose), and 2 were discarded for the reasons described above. Overall, the method appeared free of contamination caused by field sampling, laboratory storage and/or extraction, and transportation.

Table 1 shows the recovery of formaldehyde from spiked samples analyzed before, during, and after the real drinking water samples. With the exception of 1 result, the recovery of formaldehyde consistently ranged between 87 and 132%, with the analyte concentration varying between 0 and 180 μg/L. These recoveries are quite acceptable at the ppb level. The observed concentration of formaldehyde in 2 spiked samples with 0 μg/L of formaldehyde present was below the reporting limit. Furthermore, approximately 4 weeks elapsed from the time the study began until the final set of spiked samples could be analyzed. Clearly, the sample preservation procedure employed was effective; the test concentrations observed in the final set of quality assurance samples were approximately the same as those observed in the initial set.

Figures 2 and 3 present the formaldehyde concentrations from 2- and 1-story houses, respectively, for both hot and cold water. Hot and cold water samples were obtained and analyzed from each home; however, some were contaminated in the laboratory, as discussed previously, and were omitted from the figures. Several trends are readily apparent. First, 21% (4 of 19) of the cold waters and 48% (10 of 21) of the hot waters from the 2-story dwellings exceeded 20 μg/L. Second, in the 1-story houses, the concentration of formaldehyde in 78% (7 of 9) of the cold waters and 80% (8 of 10) of the hot waters exceeded 20 μg/L. Third, in most cases, the drinking water samples that exhibited strongly elevated levels of formaldehyde were hot water samples. This latter trend was less

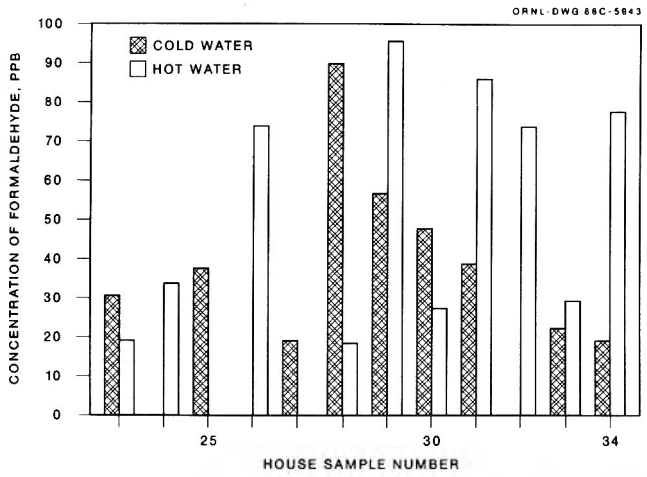

Figure 3. Formaldehyde concentrations in hot and cold water from 1-story homes. Shaded bars, cold water; empty bars, hot water.

pronounced in the 1-story homes because both hot and cold water samples frequently contained elevated levels of formaldehyde. The pair of drinking water samples from the home that had no poly(acetal) fittings (all copper plumbing) exhibited formaldehyde levels below the method reporting limit.

The data presented may be explained by considering the plumbing design in the 1- and 2-story homes. The number of poly(acetal) elbows and tees varies by perhaps 50% between the 1- and 2-story houses (12 vs 20, respectively), but the length of pipe present, and the volume of water in the plumbing system, is 2 to 3 times greater in the 2-story dwellings. Hence, the apparent reduction in the formaldehyde content for 2-story houses may be caused by dilution. In addition, some of the plumbing passes through the attic, and the resulting water temperature within the pipes is somewhat dependent on the time of year the water is sampled. Because the sampling was performed in early August, the water temperature within the pipes may have become sufficiently elevated to leach formaldehyde from elbows and tees in both the hot and cold water lines. The pipes themselves were constructed from polybutylene, not poly(acetal), and, therefore, were not a contributing factor to the formaldehyde level.

Conclusions

The analytical procedure described is clearly applicable for quantitating formaldehyde in drinking water at action thresholds above 20 μg/L, which have been set by certain state and federal agencies. The poly(acetal) fittings employed in the 1- and 2-story houses are undergoing chemical degradation and releasing formaldehyde at ppb levels into cold and hot drinking water. The concentrations are greater in hot than in cold water, and in 1-story than in 2-story houses.

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Capillary Column Gas Chromatographic Determination of Dicamba in Water, Including Mass Spectrometric Confirmation

NANCY C. JIMENEZ, YOUSEF H. ATALLAH, and THOMAS R. BADE
Sandoz Crop Protection Corp., 1300 E Touhy Ave, Des Plaines, IL 60018

A sensitive method is described for determining dicamba at low μg/L levels in ground waters by capillary column gas chromatography with electron-capture detection (GC-EC); compound identity is confirmed by gas chromatography-mass spectrometry (GC-MS) using selected ion monitoring. Dicamba residue is hydrolyzed in KOH to form the potassium salt. The sample is then extracted with ethyl ether which is discarded. The aqueous phase is acidified to pH < 1 and extracted twice with ethyl ether. The combined ethyl ether extracts are concentrated, and the residue is methylated using diazomethane to form the corresponding dicamba ester. The derivatized sample is cleaned up on a deactivated silica gel column. The methylated dicamba is separated on an SE-30 capillary column and quantitated by electron-capture or mass spectrometric detection. Average recoveries ($X \pm SD$) for ground water samples fortified with 0.40 μg/L of dicamba are 86 ± 5% by GC-EC and 97 ± 7% by GC-MS detections. The EDL (estimated detection limit) for this method is 0.1 μg dicamba/L water (ppb).

Dicamba, 3,6-dichloro-*o*-anisic acid, is a benzoic acid broadleaf herbicide. It is the active ingredient in Banvel® herbicide, a dimethylamine salt formulation, which is effective in controlling weeds infesting corn, sorghum, small grains, asparagus, sugarcane, pasture, rangeland, and non-cropland areas. Methods of dicamba residue analysis in these crops are available (1-5).

In the past 3 years, several analytical methods for the determination of dicamba in water, including a multiresidue method for the determination of both neutral and acidic herbicides (6), have been developed. Chemical derivatizations using diazomethane, pentafluorobenzyl bromide, and (2-cyanoethyl)dimethyl(diethylamino)silane have been evaluated and compared (7, 8). A liquid chromatographic LC method using on-line sample enrichment and separation has been developed (9). But due to the lower sensitivity of UV detection, the method has a detection limit of 1 ppb. An isotope dilution GC-MS technique for the detection of 0.1-1.0 ppb concentrations of dicamba in water has been investigated (10). Drawbacks of this method are the availability and cost of the stable-labeled isotopes and the time-consuming pentafluorobenzylation derivatization. Recently, a paper was published describing a procedure for the analysis of dicamba in water using solid-phase extraction and ion-pair liquid chromatography (11). However, the procedure has a detection limit of 1.6 ppb dicamba.

The objective of the present study was to develop a simple and sensitive method with detection limit of 0.10 μg/L (ppb), which could be used for qualitative and quantitative determinations of dicamba residue in ground water.

METHOD

Apparatus

(a) *Gas chromatography (GC-EC).*—Hewlett-Packard Model 5880A gas chromatograph equipped with ^{63}Ni electron-capture detector, level 2 terminal, split-splitless capillary column injection port, Model 7671A autosampler, and Varian 401 chromatography data system. Column: 30 m × 0.53 mm id crosslinked methyl silicone gum, 0.88 μm film thickness (Hewlett-Packard). Operating temperatures: injection port 250°C, detector 350°C, initial column oven 100°C, hold 0.5 min, programming rate 30°/min, hold 4.75 min at 150°C. Splitless valve on for 0.5 min. Helium carrier gas at 9.4 mL/min. Detector make-up gas, argon-methane (95 + 5) at 30 mL/min.

(b) *Gas chromatography-mass spectrometry (GC-MS).*—Hewlett-Packard Model 5880A GC equipped with Model 5970B mass selective detector (MSD), Series 200 computer, 9133 disc XV drive, split-splitless injection port, level 2 terminal, and Model 7671A autosampler. Column: 25 m × 0.2 mm id crosslinked methyl silicone gum, 0.11 μm film thickness (Hewlett-Packard), directly interfaced to electron-impact ion source. GC operating conditions: injection port 250°C, initial column oven 100°C, hold 0.5 min, programming rate 30°/min, hold 1.5 min at 180°C. Splitless valve on for 0.5 min. Helium carrier gas at 10 psi. MS operating conditions: analyzer pressure 3.5×10^{-5} torr; ionization mode, electron impact; multiplier voltage, 1600 V; dwell time, 500 ms; transfer line temperature, 190°C; data acquisition mode, selected ion monitoring (SIM) m/z 203, 205, and 234 ions, quantitation by external standard based on ion 203; data acquisition delay time, 3 min.

(c) *Glassware.*—Evaporative-concentrator: Kuderna-Danish (KD), with 250 mL flask, 12 mL receiver tube, adapter, and Vigreaux column. Funnel: 75 mm diameter, 60°.

(d) *Chromatographic columns.*—Fitted with water jacket and Teflon stopcock (15 mm id × 45 cm).

(e) *Esterification apparatus*—See Figure 1.

(f) *Nitrogen evaporator.*—N-Evap (Organomation Associates).

Reagents

(a) *Solvents.*—Acetone, ethyl ether (2% ethanol preservative), hexane, methanol, pentane. All pesticide grade or equivalent.

(b) *Reagent water.*—Distilled, deionized water.

(c) *Carbitol.*—2-(2-ethoxyethoxy) ethanol (Aldrich Chemical Co.).

(d) *Diazomethane.*—Generated by using Diazald (Aldrich Chemical Co.). *Caution*: Diazomethane is highly toxic, carcinogenic, and explosive. Prepare and handle only in high face velocity hood while wearing half-face respirator with chemical cartridge.

(e) *Hydrochloric acid solution (6N).*—Prepare by adding 500 mL concentrated HCl to 500 mL water.

(f) *Potassium hydroxide solution (6N).*—Dissolve 336 g KOH in water and dilute to 1 L.

(g) *Deactivated silica gel.*—3%. Activate silica gel 60, 70-200 mesh (EM Reagents, MC/B Manufacturing Chemists, Inc.) at 250°C for 2½ h. Deactivate adsorbent by adding 6.0 g reagent water to 194.0 g activated silica gel. Mix well on shaker for 15 h and store at room temperature in 32 oz bottle with poly-seal screw cap. Prepare fresh biweekly.

(h) *Sodium sulfate.*—Anhydrous granular (Eastman Kodak Co.).

(i) *Dicamba standard.*—2-Methoxy-3,6-dichlorobenzoic acid (99.6% purity). *(1) Stock solution.*—1 mg/mL. In 100 mL volumetric flask, dissolve 100 mg dicamba in methanol,

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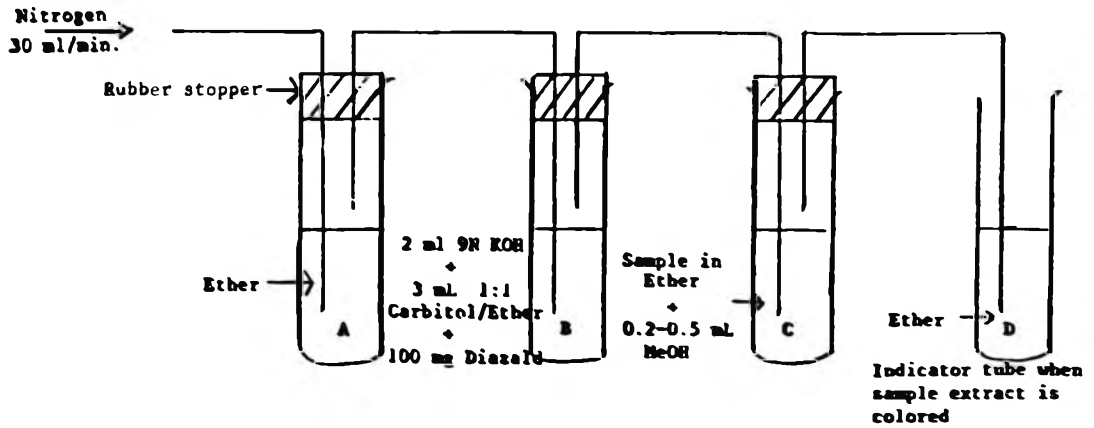

Figure 1. Esterification apparatus.

dilute to volume, and mix well. (*2*) *Dilute stock solution.*—10 μg/mL. Pipet 1.0 mL stock solution into 100 mL volumetric flask, dilute to volume with methanol, and mix well. (*3*) *Fortifying solution.*—1 μg/mL. Pipet 10.0 mL dilute stock solution into 100 mL volumetric flask, dilute to volume with methanol, and mix well. (*4*) *Working standards.*—0.1 ng/μL. Methylate 1.0 mL dilute stock solution, dilute to 100 mL with hexane, and mix well. (*5*) *Dilute working standards.*—Prepare 0.005, 0.01, 0.02, and 0.03 ng/μL working standards by appropriately diluting working standard solution with hexane.

Fortification of Water Samples

Fortify 250 mL reagent water sample with 100 μL fortifying solution. Shake and hold for 30 min before hydrolysis.

Extraction

Shake water sample (1 L), collected in 32 oz glass jars with poly-seal screw cap. Transfer 250 mL aliquot to 16 oz bottle. Add 5 mL 6N KOH solution to sample (final pH must be greater than 8). Close bottle tightly and shake 1 h using mechanical platform shaker. Transfer contents of bottle to 500 mL separatory funnel with Teflon stopcock. Add 100 mL ethyl ether and shake funnel vigorously 1 min. After phases separate, drain aqueous layer to another 500 mL separatory funnel and discard organic layer. Adjust aqueous layer to pH < 1 with 5 mL 6N HCl. Add 100 mL ethyl ether to acidic aqueous layer and shake vigorously 1 min. After phases separate, drain aqueous layer back to original separatory funnel and repeat extraction. Combine organic extracts. Discard water layer. Filter combined organic extracts through sodium sulfate to Kuderna-Danish evaporative-concentrator. Rinse sodium sulfate with 15–20 mL ethyl ether. Add 1 mL hexane and evaporate combined organic extracts to ca 1 mL in 65°C water bath.

Derivatization

Set up tubes A, B, C, and D (Figure 1). Tube A serves to saturate nitrogen flow with ether which transmits diazomethane from B (diazomethane generator) to C (sample container). Tube D is used as indicator for excess diazomethane when sample extract is colored. To tube B (20 × 150 mm test tube), add 2 mL 9N KOH solution and 3 mL carbitol-ethyl ether (1 + 1). To tube C which contains the sample extract, add 0.2–0.5 mL methanol and dilute to 5 mL with ethyl ether. To tube D, add 3 mL ethyl ether. Add ca 100 mg Diazald with spatula to tube B and seat rubber stoppers in tubes A, B, and C. Bubble nitrogen gas through solutions in A, B, C, and D at 30 mL/min. Remove tube C when solution turns yellow. For colored extracts, observe color change in tube D. (*Caution*: Diazald is a carcinogen. Gloves and half-face respirator with cartridge must be worn when working with this reagent. In addition, the diazomethane gas generated is extremely toxic and should only be used in hoods which are operating efficiently (≥ 150 LFM). Diazomethane gas may explode when heated to 100°C or when in contact with ground glass or cracked and chipped glass. Alkaline metals may cause diazomethane to explode.)

Derivatize samples in order of increasing concentration of the compound, if this is known, to prevent cross-contamination of sample. Rinse diazomethane delivery tube with ether before derivatizing next sample. Change diazomethane generating solution after every 2 samples. Deactivate diazomethane solutions by slowly pouring into large quantity of water. Dispose of waste solution in compliance with federal, state, and on-site safety regulations and procedures.

Cap KD tube for 15 min (Teflon stopcocks are used for this purpose). If solution remains yellow throughout this interval, evaporate sample to ca 1 mL under gentle nitrogen stream in a hood with face velocity ≥ 150 LFM. Do not use heat for this procedure. If, however, yellow color disappears before 15 min, recharge sample extract with diazomethane. Dilute methylated extract to 5 mL with hexane for GC-EC or GC-MS quantitation if column cleanup is not necessary.

Column Cleanup

Set up jacketed columns. Fit each with glass wool plug to contain silica gel, and open stopcock. To 250 mL separatory funnel, add 70 mL ethyl ether–pentane eluant (5 + 95) and 20 g 3% water-deactivated silica gel. Shake well and quickly transfer slurry to column. Rinse the separatory funnel with 10 mL eluant and add rinse to column. Cover silica gel bed with 1 cm granular Na_2SO_4 and drain solvent just to Na_2SO_4 layer. Add 5 mL eluant to methylated sample and transfer to silica gel column. Rinse KD with two 5 mL portions of eluant. Successively apply rinses to column and let each rinse penetrate to top of bed. Pass another 55 mL eluant through column and discard. Elute and collect methylated dicamba residue in next 85 mL eluant. Add 5 mL hexane and concentrate sample to 5 mL on 65°C water bath for GC-EC or GC-MS quantitation.

Analysis

Inject 2 μL working standard solutions or sample extracts in splitless mode and quantitate by external standard method.

Prepare standard curve for dicamba by injecting known amounts of standard and plotting peak height vs concentra-

Table 1. Recoveries after column cleanup from ground water samples fortified at 0.40 ppb

Sample	Recovery, %[a]	
	GC-EC	GC-MS
860-05690	85	98
860-05691	80	87
860-05692	90	100
860-05693	90	104
Mean, %	86	97
SD	5	7

[a] Results corrected for blank.

Table 2. Retention times and responses of potential dicamba interferences considered to leach into ground water

Chemical group	Pesticide	Retention time, min	Response (height counts)[a]
1. Herbicides			
Benzoid acids	dicamba	5.792	24810
	5-hydroxy dicamba	—	ND
	3,5-dichlorobenzoic acid	4.104	17102
Acetanilides	alachlor	—	ND
	propachlor	—	ND
Triazines	atrazine	—	ND
	cyanazine	—	ND
	metribuzin	—	ND
Chloro and	dinoseb	—	ND
dinitro phenols	pentachlorophenol	6.188	4907
Chlorophenoxy	2,4-D	—	ND
acids	dichlorprop	—	ND
Diphenamid	diphenamid	—	ND
Picolinic acids	picloram	—	ND
Thiocarbamates	butylate	—	ND
Others	trifluralin	—	ND
2. Fungicides			
Others	chlorothalonil	—	ND
3. Insecticides			
Organophosphorus	phorate	—	ND

[a] ND = not detected. Although the compound has GC-EC sensitivity it did not elute during the chromatographic run. All acids and phenols were methylated.

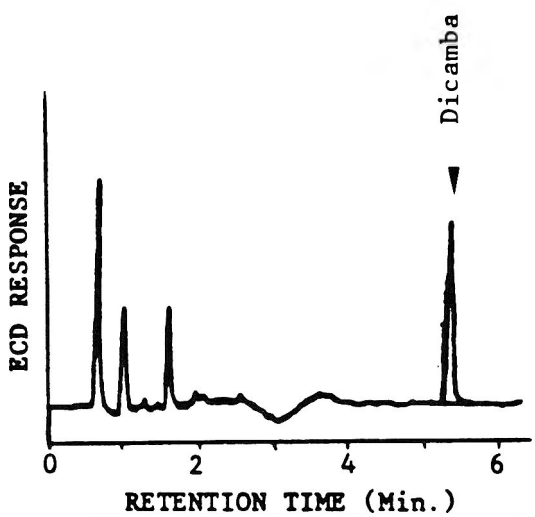

Figure 2. ECD-gas chromatogram of standard dicamba (0.01 ng acid equivalent/μL).

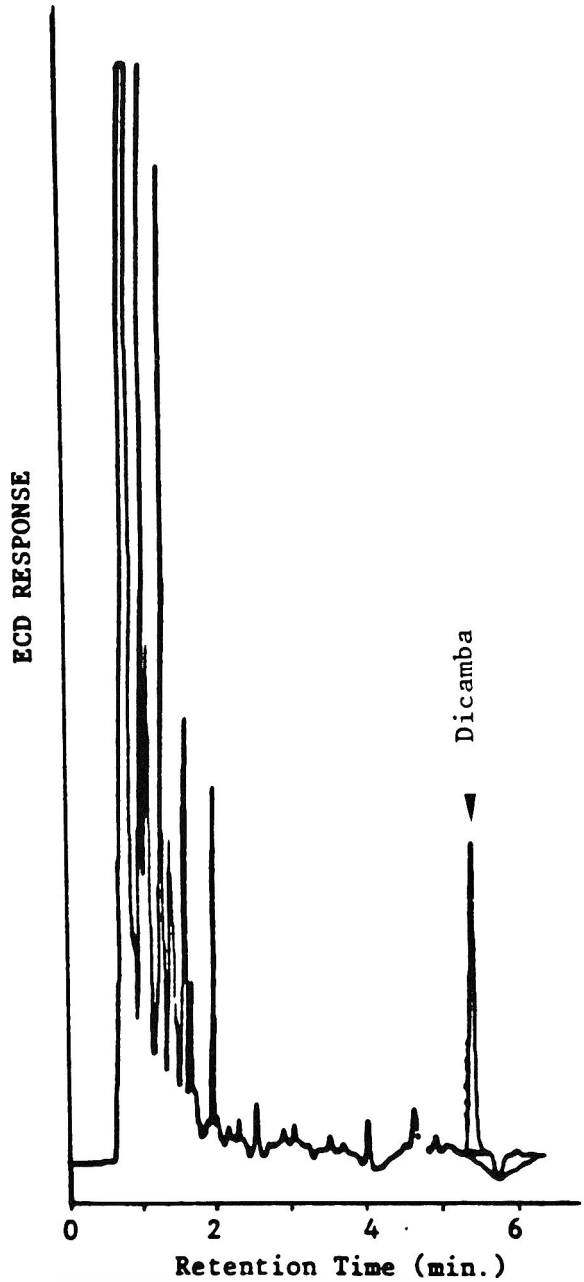

Figure 3. ECD-gas chromatogram of ground water sample, fortified with 0.40 ppb dicamba.

tion of standard (ng/μL) on log-log paper. Determine concentration of analyte (ppb) in aliquot of injected sample from peak height and standard curve. Calculate concentration of residue in sample, using following expression:

$$ppb = (C_e \times V_s \times 1{,}000)/W$$

where ppb = concentration of analyte in sample in parts per billion (ng/g); C_e = concentration of extract (ng/μL) determined from standard curve; V_s = volume of final sample extract (mL), taking into account all dilutions; W = weight of sample (g) taken for analysis.

Results and Discussion

Detection Limit

The EDL (estimated detection limit) of the method is 0.1 μg/L (ppb). The value was determined by multiplying the standard deviation of replicate measurements of samples fortified at 0.4 ppb dicamba (0.0192) by the Student's *t*-

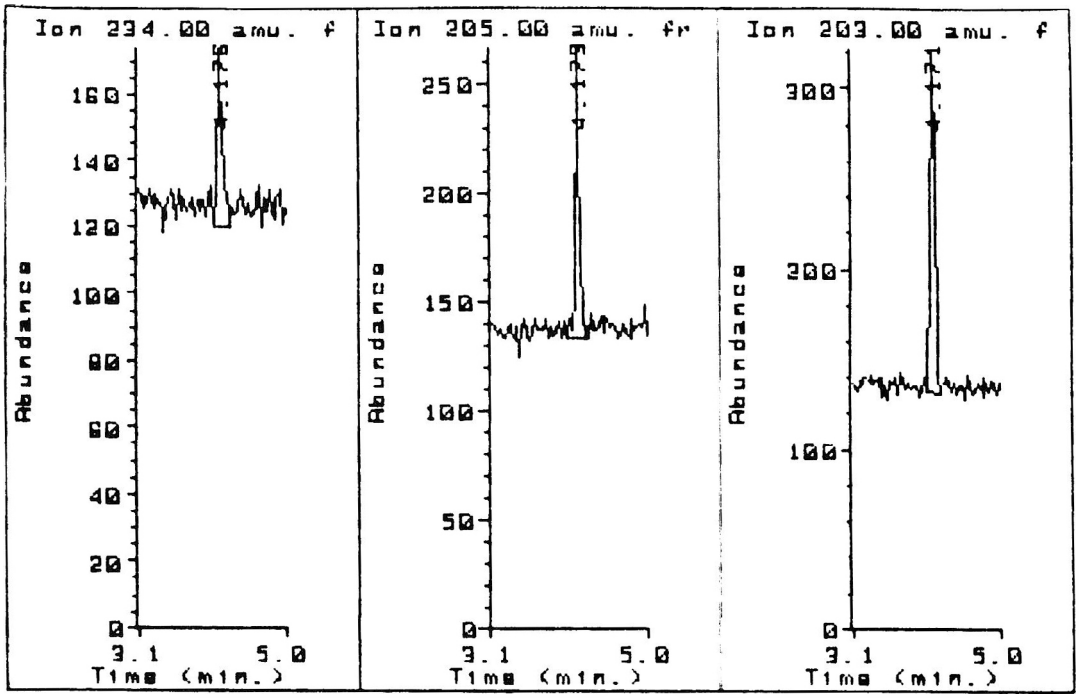

Figure 4. MSD-gas chromatogram of standard dicamba (0.01 ng acid equivalent/μL).

value appropriate for a 99% confidence level and a standard deviation estimate with n − 1 degrees of freedom (5.841).

Recovery and Precision

To determine the method recovery and precision, replicate reagent water samples were fortified with 0.10 and 0.40 μg/L dicamba. The water samples were alkaline-hydrolyzed, acidified to pH < 1, and extracted with ethyl ether. The dicamba in the extracts was derivatized to its ester. No column cleanup was used. Average recoveries ($X \pm$ SD) were 84 ± 14% at 0.10 ppb (96, 104, 76, 80, 68, and 78%) and 92 ± 9% at 0.4 ppb (100, 105, 82, 80, 82, 102, 90, 90, and 95%).

This method was also validated with ground water samples from Clayton, North Carolina, at a fortification level of 0.40 μg/L. The analyses were conducted as with reagent water samples except the samples were subjected to silica gel column cleanup. Average recovery ($X \pm$ SD) was 86 ± 5% (see Table 1).

A trace amount (0.04 μg/L) of material, interfering with dicamba detection, was found in ground water samples; the recoveries in these samples were corrected for this interference.

Quality Assurance

Method interferences may be caused by contaminants in solvents, reagents, and glassware. A method blank must be

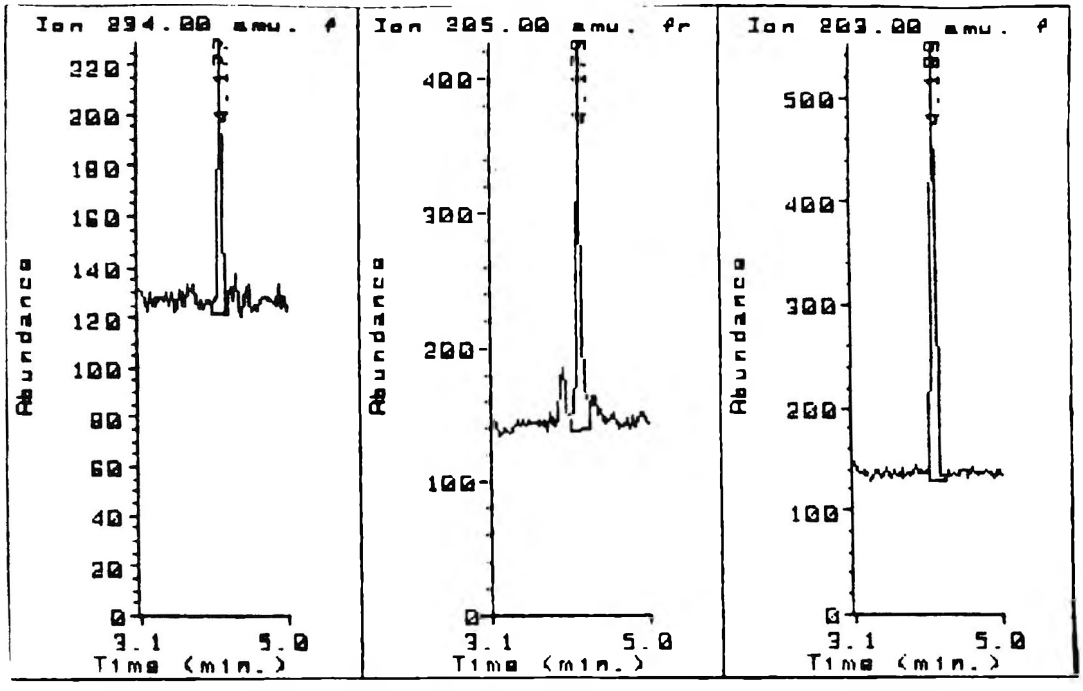

Figure 5. MSD-gas chromatogram of ground water sample, fortified with 0.40 ppb dicamba.

run routinely to monitor for interferences.

Glassware must be thoroughly cleaned by washing with hot water and detergents followed by rinsing with tap water and deionized water. Glassware should be dried at room temperature or in an oven set at 120°C, and finally rinsed with acetone. Reagents and solvents must be of high purity grade. Plastics should not be used since interferences from phthalate esters may result. Representative water samples should be collected in 32 oz glass jars. Sample cross-contamination during quantitation can be minimized by between-sample rinsings of the syringe with hexane and the sample extract to be injected. Matrix interference can be minimized by solvent partition and silica gel column cleanup as described above.

Some chemical compounds that are considered to leach into ground water might also interfere in the GC-EC analysis of dicamba. A GC standard solution, 0.1 ng/μL each of the compounds listed in Table 2 was injected into the GC-EC using the parameters described in this method even though they might not be recovered by this method. None of these compounds interfered with dicamba under the GC conditions used. Representative GC-EC chromatograms of dicamba reference standard (0.10 ng/μL) and ground water sample fortified at 0.40 μg/L dicamba are shown in Figures 2 and 3.

Confirmation

Identification of pesticides in water even at trace levels is a serious public concern, thus confirmation of a chromatographic peak as the compound of interest is a serious matter and should be included in analytical methods addressing pesticide residue in water. Dicamba residues were confirmed by GC-MS. In addition to confirmation of compound identity, the mass selective detector is used as a complement to the electron-capture detector for the quantitative analysis of dicamba.

Autotune values were used for the repeller, lenses, and electron multiplier voltages. Data were acquired in SIM mode monitoring ions 234, 205, and 203. Quantitation was done by the external standard routine based on ion 203. Identification is based on ratios of ions 234 and 205 to 203.

The mass spectrometer results agreed very well with those obtained with the electron-capture detector (see Table 1). Average recovery ($X \pm$ SD) was 97 ± 7%.

Representative GC-MS chromatograms of dicamba reference standard (0.01 ng/μL), and fortified ground water sample (0.40 μg/L dicamba), are shown in Figures 4 and 5.

Acknowledgments

The authors acknowledge the late William Cahill for his valuable advise on this project.

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Determination of Carbofuran and Its Metabolites in Rice Paddy Water by Using Solid Phase Extraction and Liquid Chromatography

KEITH W. BEAUCHAMP, JR, DAVE D. W. LIU, and EDWARD J. KIKTA, JR
FMC Corp., Agricultural Chemical Group, PO Box 8, Princeton, NJ 08543

A procedure for the determination of carbofuran and its metabolites (carbamate and phenolic) in rice paddy water is described. Water samples are concentrated on a C-18 solid phase extraction (SPE) column and eluted with methanol–water. The eluate is analyzed by reverse-phase liquid chromatography (LC) and measured by a wavelength programmable ultraviolet (UV) detector. The limit of detection for the method is 0.4 μg/L. Recovery studies were carried out at levels ranging from 1 to 15 μg/L in both rice paddy water and distilled water; recoveries ranged from 85.9 to 112.9%.

Furadan® insecticide-nematicide containing the active ingredient carbofuran (2,3-dihydro-2,2-dimethyl-7-benzofuranyl-*N*-methyl carbamate), is a broad-spectrum pest control agent used on a variety of crops. Methodology has been developed for the determination of carbofuran and its carbamate and phenolic metabolites in rice paddy water. The carbamate metabolites are 3-hydroxy carbofuran (2,3-dihydro-2,2-dimethyl-3-hydroxy-7-benzofuranyl-*N*-methyl carbamate) and 3-keto carbofuran (2,3-dihydro-2,2-dimethyl-3-oxo-7-benzofuranyl-*N*-methyl carbamate). The phenolic metabolites are 3-hydroxy-7-phenol (2,3-dihydro-2,2-dimethyl-3,7-benzofurandiol), 3-keto-7-phenol (2,3-dihydro-2,2-dimethyl-3-oxo-7-benzofuranol), and 7-phenol (2,3-dihydro-2,2-dimethyl-7-benzofuranol) (Figure 1).

Carbamate residue analysis by gas chromatography (GC) has traditionally fallen into 2 categories: direct analysis using nitrogen-sensitive detectors (1–3) and derivatization followed by electro-capture detection (4–6). The nitrogen-sensitive detectors offer high sensitivity in addition to some selectivity for carbamates. However, they lack the sensitivity required for the phenolic metabolites. Derivatization procedures introduce additional steps and often require separation of the carbamates from the phenolics before derivatization.

A GC/mass spectrometric (MS) method (7) has been reported for determination of carbofuran in the form of the 7-phenol metabolite. The phenolic metabolites of carbofuran have been determined by GC/MS either directly (8) or via derivatization (9).

Liquid chromatographic (LC) analyses of carbamates, that use both pre-column (10, 11) and post-column (12) derivatization and nonderivatization procedures (10, 13), have been reported. Carbamate and phenolic metabolites can be separated by either gradient or isocratic reverse-phase LC (14). An LC procedure using on-line trace enrichment was reported for the determination of carbofuran and its carbamate and phenolic metabolites in drinking water (15). However, the trace enrichment method is suitable only for relatively clean samples and cannot separate 3-keto-carbofuran from 3-keto-7-phenol. We herein describe an analytical procedure that can directly analyze the carbamates and phenolics in water matrixes simultaneously. The procedure utilizes solid-phase extraction for sample cleanup/enrichment and reverse-phase LC with wavelength programming for quantitation. This method demonstrates good recoveries, selectivity, and sensitivity (0.4 μg/L) and is simple and quick to perform.

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METHOD

Reagents

(a) *Reagents.*—J.T. Baker acetonitrile and methanol, LC grade; Millipore Organix Q water; and J.T. Baker concentrated phosphoric acid, reagent grade.

(b) *Solutions.—Acidified methanol.*—Add 5 drops of concentrated phosphoric acid to 1 L methanol. *Acidified water.*—Add 5 drops of concentrated phosphoric acid to 1 L water. *Acidified methanol–acidified water (60 + 40).*—Add 400 mL acidified water to 600 mL acidified methanol, and mix.

(c) *Analytical standards.*—Carbofuran, carbamate metabolites, phenolic metabolites (FMC Corp., Manufacturing Technical Support, 100 Niagara St, Middleport, NY 14105).

(d) *Standard solutions.—Primary standard solutions.*—Weigh 10 mg of each analytical standard to nearest 0.01 mg and dilute each with 20.0 mL acidified methanol. Store solutions in refrigerator. *Working standard solutions.*—Mix and dilute each primary standard with acidified methanol–acidified water (60 + 40) to achieve necessary concentration.

Apparatus

(a) *Solid-phase extraction (SPE) columns.*—6 mL C-18, high capacity (J.T. Baker Co.).

(b) *SPE vacuum manifold.*—Supelco SPE vacuum manifold (Supelco, Inc.), with vacuum pump.

(c) *Centrifuge tubes.*—10 mL, graduated.

(d) *Chromatographic system.*—Varian 5000 liquid chromatograph with built-in block contact column heater, Kratos 783 programmable wavelength detector, Spectra Physics

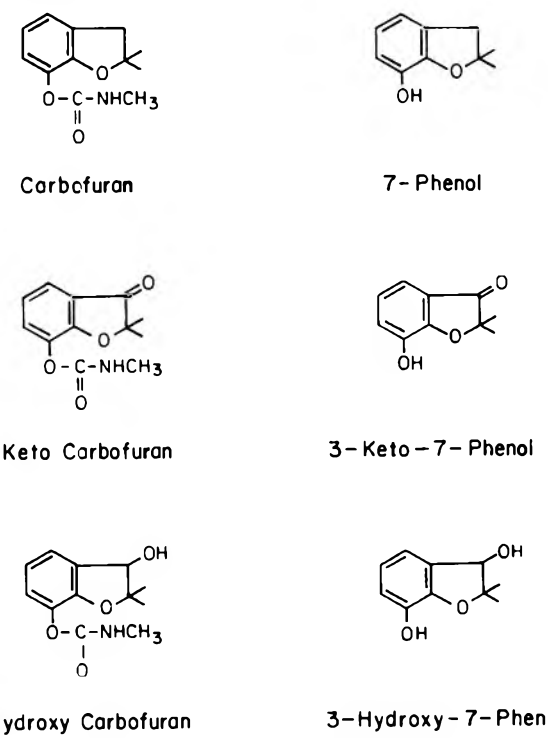

Figure 1. Structures of carbofuran and its metabolites.

Table 1. Wavelengths and retention times for LC analysis of carbofuran and metabolites

Analyte	Wavelength, nm	Retention time, min
3-Hydroxy-7-phenol	280	8.2
3-Hydroxy carbofuran	280	12.9
3-Keto-7-phenol	265	16.4
3-Keto carbofuran	250	20.6
7-Phenol	220	21.8
Carbofuran	220	25.7

Table 3. Recovery data—5 spiking level averages[a] for rice paddy water

Analyte	Av., %	CV, %
3-Hydroxy-7-phenol	100.0	11.6
3-Hydroxy carbofuran	96.8	13.1
3-Keto-7-phenol	106.4	14.5
3-Keto carbofuran	109.0	11.1
7-Phenol	103.8	13.8
Carbofuran	106.2	11.2

[a] One spiking each at 5 different levels.

4270 integrator, Varian 8055 autosampler with Rheodyne 7125 valve and 20 μL loop, and ES Industries cyclohexyl column (250 mm × 4.6 mm, 5 μm).

Solid-Phase Extraction

Attach C-18 SPE column(s) to vacuum manifold, and attach 75 mL reservoir(s) to column(s). Rinse column with 6 mL methanol followed by two 6 mL aliquots of water. Pass 100 mL sample through column under vacuum. When sample is completely aspirated, rinse reservoir with 10 mL acidified water and aspirate. Remove reservoir and rinse extraction column with 6 mL acidified water. Aspirate 15 min to dryness.

Place graduated centrifuge tube in manifold basin for collection. Pipet 1.2 mL acidified methanol onto extraction column. Let acidified methanol stand 2 min on column before applying vacuum, and collect eluate. Without removing centrifuge tube, pipet 0.8 mL acidified water onto extraction column, apply vacuum, and collect eluate. Dilute eluate to 2 mL mark with acidified methanol–acidified water (60 + 40). (Acidified water is added to ensure that final solution contains ca 40% water. Chromatographic peak efficiency is greatly affected by solvent composition of sample solution.) Analyze samples by liquid chromatography, and quantitate by external standard and peak height measurements.

Analysis

LC operating conditions: flow 1 mL/min; column temperature 40°C; and injection size 20 μL. Use 2 eluants, water (A) and acetonitrile (B), in elution gradient: 13% eluant B to 20% eluant B in 5 min, to 28% eluant B in 12 min, to 35% eluant B in 4 min, and hold for 8 min. Then wash column with 100% eluant B for 5 min, and return to initial conditions with an equilibration time of 12 min. Table 1 lists wavelengths and retention times for each analyte.

Results and Discussion

Initially, this investigation set out to develop a method with 4 goals in mind: (*1*) to determine both carbamates and phenols in the same method; (*2*) to achieve sensitivities as low as 1 μg/L for each analyte; (*3*) to be efficient (time, personnel, and equipment); (*4*) and to achieve recoveries equivalent to or better than previous work. The approach was based on previous work (unpublished) done in this laboratory utilizing SPE with carbofuran and 7-phenol in well water and wastewater. LC was chosen because gas chromatography, as conventionally practiced, does not directly provide the sensitivity required for the determination of phenols.

The chromatographic system utilized a multilevel gradient that was optimized to separate the analytes from interferences in the rice paddy water. The chromatographic conditions may be optimized to fit a particular sample matrix. The UV detector employed 4 different wavelengths, which were chosen on the basis of the absorbance of both the analytes and the matrix interferences.

The limit of detection (LOD) and the limit of quantitation (LOQ) were set for the method rather than each analyte. This was done by calculating LOD and LOQ on the least sensitive of the 6 analytes, 3-hydroxy-carbofuran. The LOD (3 times baseline noise) was determined to be 0.4 μg/mL. The LOQ (10 times base noise) was determined to be 1.4 μg/mL.

Two recovery studies were performed by spiking water samples with the 6 analytes of interest, prior to the solid phase extraction. The first recovery study was done at 2 levels (1 and 10 μg/L) in 2 matrixes (rice paddy water and distilled water). These recoveries are reported in Table 2 and are based on 4 replicate spikings in each matrix. The second study involved one spiking each at 5 different levels in rice paddy water. The averages of the 5 levels are reported in Table 3. The data in the 2 studies are consistent with each other. The rice paddy water came from untreated test plots in Louisiana and California and sampling times ranged from 8 h to 30 days after flooding. Figures 2 and 3 show a 14-day rice paddy water sample unspiked and spiked at 10 μg/L, respectively. The signal spikes and baseline level changes at approximately 15.3, 17.5, and 21.5 min are a result of programmed wavelength changes followed by autozeroing.

Table 2. Recovery data—comparison for rice paddy water vs distilled water[a]

	Rice water				Distilled water			
	1 μg/L		10 μg/L		1 μg/L		1 μg/L	
Analyte	Av., %	CV, %	Av., %	CV, %	Av., %	CV, %	Av., %	CV, %
3-Hydroxy-7-phenol	105.9	6.7	101.7	3.9	104.9	5.5	110.0	5.2
3-Hydroxy carbofuran	103.8	4.9	111.3	2.6	112.9	4.3	109.2	7.8
3-Keto-7-phenol	109.0	3.0	109.7	1.9	104.7	4.2	108.7	7.8
3-Carbofuran	98.2	4.5	107.1	3.4	109.6	4.3	109.9	8.5
7-Phenol	102.4	6.4	98.9	4.7	107.6	4.0	102.5	9.5
Carbofuran	85.9	11.1	105.3	1.9	103.1	2.9	98.8	8.8

[a] Four replicate spikings for each matrix.

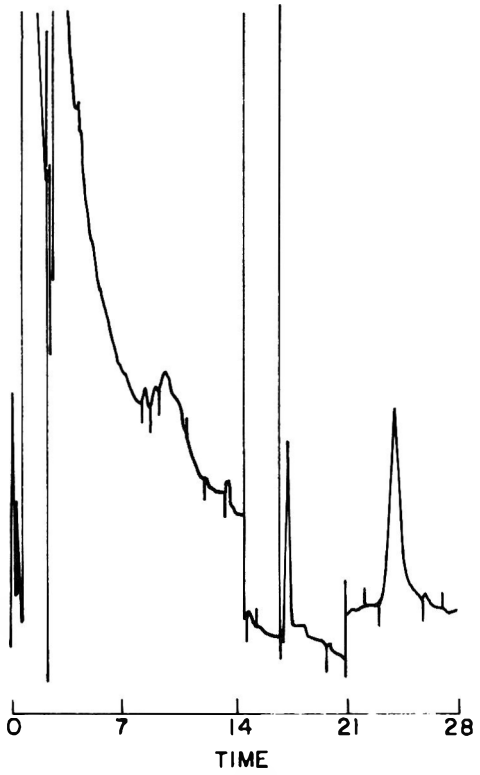

Figure 2. Rice paddy water—14 days control.

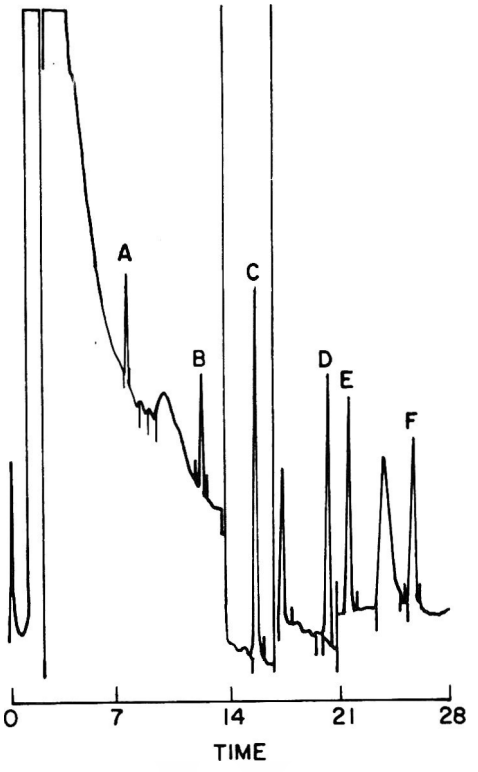

Figure 3. Rice paddy water—14 days control, spiked at 10 ppb levels (10 ng injection): A, 3-hydroxy-7-phenol; B, 3-hydroxy carbofuran; C, 3-keto-7-phenol; D, 3-keto carbofuran; E, 7-phenol; F, carbofuran.

Conclusion

This method is economical, efficient, and reliable for the determination of carbofuran and its metabolites in simple water matrixes. It offers the ability to analyze both phenols and carbamates by using one procedure. It provides a viable alternative method that alleviates the problems associated with traditional liquid/liquid extractions and derivatization procedures.

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TECHNICAL COMMUNICATION

Liquid Chromatographic Determination of Epimerization of Chlortetracycline Residue to 4-epi-Chlortetracycline Residue in Animal Feed, Using McIlvain's Buffer as Extractant

ELIZABETH E. MARTINEZ and WILBERT SHIMODA
Food and Drug Administration, Animal Drug Research Center, Denver Federal Center, Denver, CO 80225

A method for studying McIlvain's solution as a factor in epimerization of chlortetracycline in animal feed has been developed. McIlvain's solution (McBuffer) was used previously to extract oxytetracycline (OTC), tetracycline–HCl (TC-HCl), and chlortetracycline–HCl (CTC-HCl) from animal feed because it gave the best recoveries for all 3 tetracyclines; however, the McBuffer solution caused epimerization of CTC-HCl to 4-epi-chlortetracycline (4-epi-CTC). Recovery results were 30–40% lower for CTC than for OTC and TC from the same sample extract. The levels of concentration tested for CTC and 4-epi-CTC were 10 ppm, the detection limits for both were 3 ppb, and the calibration curves were linear between 5 and 80 ppm for both CTC-HCl and 4-epi-CTC. The present study shows that CTC recoveries averaged 35% lower because of epimerization initiated by the use of McBuffer in the extraction procedure.

Studies have been conducted on the epimerization of chlortetracycline (CTC) to 4-epi-chlortetracycline (4-epi-CTC) since 1957. McCormick et al. (1) used reference standard CTC to study the reversible epimerization of compounds in the tetracycline (TC) family. Howell et al. (2) developed a liquid chromatographic (LC) method to determine CTC in premixes; they also studied the formation of CTC epimers and their importance in the determination of CTC in feeds and premixes. Katz and Fassbender reported on the epimerization of CTC in mixed feeds (3) and on fat as a factor in the epimerization of CTC in feeds (4). Epimerized, dehydrated, and epimerized-dehydrated forms of TC have been shown to be impurities of CTC (5).

Methods have been developed to determine CTC in feeds (6), premixes (7), tissues (8), water (9), and other matrixes. The antibiotic has been separated by liquid chromatography and detected by ultraviolet (2, 10), electrochemical (11), and mass spectroscopic (9) methods. High performance reverse-phase thin-layer chromatography (TLC) with colorimetric detection (12), TLC with flame ionization (13) or fluorometric (14) detection, electrophoresis (15), and microbiological (16) and turbidimetric (8) methods have also been used for CTC determination in feeds. None of these reports discuss the possible interference of 4-epi-CTC in the determination of CTC residues in animal feeds.

The method reported here was developed to show that the use of McBuffer causes the epimerization of CTC of 4-epi-CTC, which, in turn, causes significantly lower recoveries of CTC from the animal feed extracts. As reported in a previous method developed by Martinez & Shimoda (17), McBuffer did not appear to affect the recoveries of oxytetracycline (OTC) and TC to any discernable degree.

METHOD

Reagents

See *Reagents*, ref. 17, **(a)**–**(g)** (*1*)(*2*), **(h)**, and **(i)** plus the following:

(j) *Reference standards.*—Label declaration (μg/mg): CTC-HCl 1000 (U.S. Pharmacopeial Convention, Inc., 12601 Twinbrook Pkwy, Rockville, MD 20852); 4-epi-CTC 1000 (American Cyanamid Co., Pearl River, NY 10965). (*1*) *Stock solution.*—CTC-HCl plus 4-epi-CTC, 1000 μg/mL. Weigh 10 mg each CTC-HCl and 4-epi-CTC, combine in 10 mL volumetric flask, and dilute to volume with anhydrous methanol. Store in cool place. Discard after 10 days. (*2*) *Working solution.*—10 μg/mL. Pipet 1.0 mL stock solution (*1*) into 100 mL volumetric flask and dilute to volume with LC mobile phase. Prepare daily.

Apparatus

See *Apparatus*, ref. 17, **(a)**–**(f)**, **(h)**, and **(i)** plus the following:

(g) *Liquid chromatograph.*—Hewlett-Packard Model 1090, equipped with built-in diode-array detector, variable volume injector, automatic sampling system, analytical work station, and Think Jet Printer (Hewlett-Packard, Englewood, CO 80110). Operating conditions: eluant at room temperature, flow rate 0.75 mL/min; injection volume 10 μL; sample wavelength 370 nm; reference wavelength 550 nm; threshold 1.5 mAU; range 4.0 mAU full scale; peak width 0.15 min; stop time 5.3 min; sample interval 640 ms; spectrum range from 210 to 400 nm; time window 10 min and zero at 10%; LC mobile phase (see *Reagents*, **(f)**).

Sample Preparation

Weigh 10 g finished feed into 250 mL glass centrifuge bottle with ground-glass stopper. Add 50 mL McBuffer (pH 2.0). Shake vigorously 20 min on mechanical shaker. Centrifuge mixture 15 min at 2000 rpm. Decant extract into 150 mL beaker. Repeat extraction with additional 50 mL McBuffer (pH 2.0). Combine extracts and mix. Continue sample preparation procedure as described in ref. (17); use 100 mL pear-shape flask instead of 150 mL pear-shape flask, and use reference standard mixture that contains 10 μg/mL each of CTC-HCl and 4-epi-CTC to compare with unknown results.

Results and Discussion

This method was developed as a consequence of previous research (17) on determining TC-HCl, OTC, and CTC-HCl residues in animal feeds. Martinez and Shimoda (17) observed that the extraction procedure used produced an unknown peak that appeared between peaks 2 and 4 (Figure 1). The presence of this peak appeared to correspond to a reduction of CTC-HCl recovery, which was consistently 30–40% lower than those for OTC and TC. When we were able to obtain a 4-epi-CTC reference standard, the McCormick method (1) was used to identify the product as 4-epi-CTC, an epimer of CTC-HCl. The chemical structures of CTC-HCl and 4-epi-CTC are shown in Figure 2.

A CTC-HCl reference standard was analyzed at 1, 4, and 24 h after extraction by the McCormick method to determine the extent of epimerization. The chromatograms (not shown) indicated that CTC-HCl and 4-epi-CTC attained equilibrium between 1 and 4 h; however, the peak heights of the 2 compounds were reversed after 4 h from what they were

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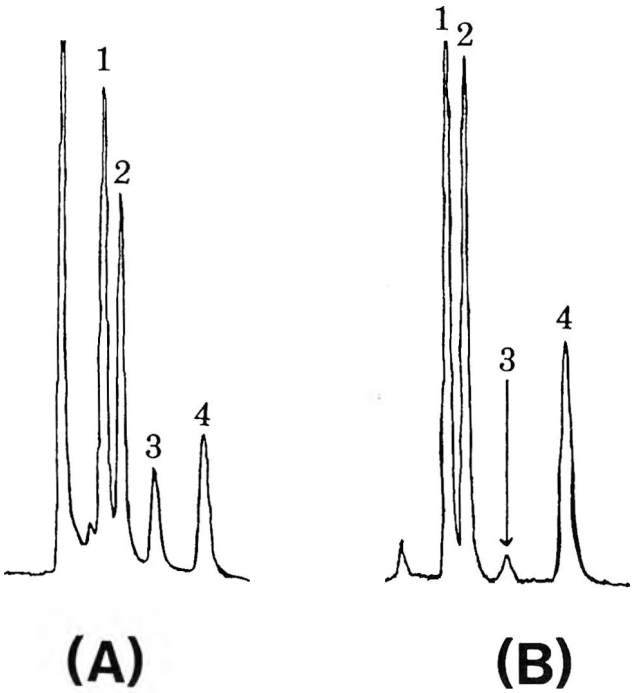
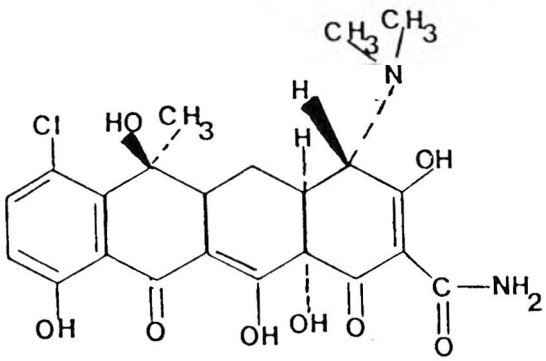


Figure 1. Chromatograms of (A) conventional chick feed spiked with 10 μg/g each of (1) OTC, (2) TC-HCl, (3) peak unknown, and (4) CTC-HCl, and (B) reference standard mixture containing 10 μg/mL each of (1) OTC, (2) TC-HCl, (3) peak unknown, and (4) CTC-HCl.

initially. The peak height of 4-epi-CTC appeared greater than that of CTC-HCl.

To determine the extent of epimerization of CTC-HCl in feeds extracted with McBuffer, we used conventional chick feed obtained from the Center for Veterinary Medicine



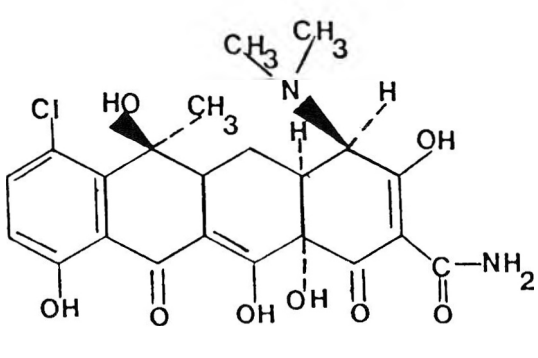


Figure 2. Structures of CTC-HCl and 4-epi-CTC.

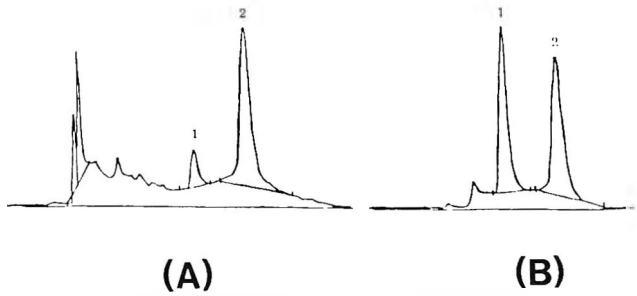


Figure 3. Chromatograms of (A) conventional chick feed spiked with 10 μg/g each of (1) trace 4-epi-CTC and (2) CTC-HCl, and (B) reference standard mixture containing 10 μg/mL each of (1) 4-epi-CTC and (2) CTC-HCl.

(CVM). We prepared 3 sets of feed samples fortified with 10 μg/g each of CTC-HCl, 4-epi-CTC, and CTC-HCl plus 4-epi-CTC. The extraction procedure was essentially the same as that of Martinez and Shimoda (17), except that this extraction procedure used back-to-back 50 mL extractions using McBuffer (pH 2.0), because we found that CTC recovery was increased an average of 20% by using a more acidic solution of McBuffer. We also found that there was a definite correlation between the use of a highly acidic solution of McBuffer, the epimerization process, and the increased recoveries of CTC-HCl from animal feed extracts. Figure 3 shows chromatograms of feed extract containing CTC-HCl only and a reference standard containing CTC-HCl plus 4-epi-CTC. Peak 1 is the epimer of CTC-HCl; peak 2 is CTC-HCl. (Peak integration and calculations shown were performed by the LC system. A linear regression analysis was performed from 4 different reference standards, and concentrations of unknowns were obtained from this curve.)

Results obtained for CTC-HCl, 4-epi-CTC, and CTC-HCl plus 4-epi-CTC for the 3 sets of samples analyzed are shown in Table 1. The samples were analyzed at 3 different time intervals to show the extent of epimerization. The data show that epimerization occurs during the initial extraction; the solution was analyzed 4 h after the initial extraction. It also shows that the feed extracts spiked with CTC-HCl or 4-epi-CTC only appeared to reach a level at which both compounds attained their maximum peak; the sample spiked with a combination of both these compounds showed that CTC-HCl continued to increase in concentration after 6 days. The reason for the increase in CTC is unknown. This sample was not analyzed after 6 days. The analyses performed 7 days after the initial extraction of CTC-HCl and 6 days after the initial extraction of 4-epi-CTC show the highest recoveries for these 2 samples.

The study also revealed at least 2 reasons for the low

Table 1. Average percent recovery for 3 sets of 5 feed samples fortified with 10 μg/g of CTC-HCl, 4-epi-CTC, and CTC-HCl plus 4-epi-CTC analyzed at 2 time intervals after initial analysis[a]

CTC-HCl		4-epi-CTC		CTC-HCl	4-epi-CTC
initial extract		initial extract		initial extract	
CTC[b]	epi-CTC	CTC[c]	epi-CTC	CTC[d]	epi-CTD
47	11	6	15	40	25
7 days post		6 days post		4 days post	
46	20	25	55	83	59
9 days post		8 days post		6 days post	
40	21	18	38	105	47

[a] Results based on average percentage of ppm recovered from 5 samples.
[b] Feed sample spiked with CTC-HCl.
[c] Feed sample spiked with 4-epi-CTC.
[d] Feed sample spiked with CTC-HCl plus 4-epi-CTC.

recovery of CTC-HCl from animal feed using the method developed by Martinez and Shimoda (17). The first was epimerization of CTC-HCl in CTC-fortified feed extracts when McBuffer solution was the extractant, and the other is that the amount of 4-epi-CTC produced in feed extracts increases with time. All samples were refrigerated between analyses to keep the sample from undergoing further reaction. Blank control samples did not show any visible peaks at any time. The results reported as percent recovery for all data in Table 1 are reproducible.

The purpose of this study was to determine the cause of lower recovery data for CTC-HCl obtained in a previous study in which CTC-HCl was one of 3 drugs of interest. We used CTC-HCl and 4-epi-CTC spiked feed and pH 2.0 McBuffer as the extractant to obtain data to address the problem. The choice of pH 2.0 McBuffer may not have been as suitable as some CTC extracting solutions mentioned in the literature, but we were investigating the reason for the low recovery of CTC-HCl from a previous study in which 2 of the 3 drugs of interest gave very good recoveries using McBuffer as the extractant. We changed the initial procedure to a pH 2.0 McBuffer double extraction instead of pH 4.5 to 2.0.

Certain factors should be considered when this procedure is used to analyze more than one TC when CTC-HCl is present in the feed sample: (*1*) the chemical structure of each drug of interest and how each will react to the extraction solution, (*2*) the interference of other drugs in animal feed with TCs that can be extracted with McBuffer, and (*3*) the epimerization of CTC-HCl to 4-epi-CTC. This epimerization needs to be considered in order to obtain more realistic analytical results of CTC-HCl residues in animal feed extracts.

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SYMPOSIUM ON FERTILIZERS: PHOSPHATE EVALUATION AND ANALYSIS

102nd Annual International Meeting of AOAC, August 1988

The lack of interlaboratory precision in the determination of phosphorus in fertilizers led to a meeting of fertilizer chemists in Atlanta, GA, in 1880. Subsequent meetings of this group generated the organization of AOAC in 1884. The first committee and referee appointed at the initial meeting of AOAC were asked to investigate methods to determine phosphorus in fertilizers. The subject has continued uninterrupted as an AOAC activity to the present day. At times, it has sparked controversy, but it has also been influential in regulating national and international trade of this important product.

This symposium was organized by the General Referee for Fertilizers and Limiting Material to highlight some of the current investigations on phosphorus methodology. It also provided a forum for several scientists from the National Fertilizer Development Center to present results from their individual studies.

The eight presentations of the symposium reported on (*1*) chemical characterization and bioavailability of the water-insoluble fraction of monoammonium phosphate; (*2*) a new method to determine phosphorus, sulfur, and metals by inductively coupled plasma spectrometry; (*3*) a comparison of water-soluble phosphorus determined by AOAC and ISO (International Organization for Standardization) methods; (*4*) a new phosphate rock standard recently issued by the National Institute of Standards and Technology; (*5*) an automated method for phosphorus with comparative data to the official AOAC method; (*6*) recalculated statistical parameters on past collaborative studies including some on phosphorus in fertilizers; (*7*) the application of the official AOAC method for available phosphorus in bone meal; and (*8*) a mechanized system to extract available phosphorus from fertilizers. Six of the eight papers are included in the following collection. Papers *4* and *6* will be published at a later date.

FRANK J. JOHNSON
National Fertilizer Development Center
Tennessee Valley Authority
Muscle Shoals, AL 35660

Chemical Characterization and Bioavailability of Phosphorus in Water-Insoluble Fractions of Three Mono-Ammonium Phosphate Fertilizers

F. J. SIKORA, E. F. DILLARD, and J. P. COPELAND
Tennessee Valley Authority, F242 NFDC, Muscle Shoals, AL 35660
G. L. MULLINS
Auburn University, Alabama Agricultural Experiment Station, Department of Agronomy and Soils, Auburn, AL 36849

Elemental impurities in mono-ammonium phosphate (MAP) fertilizers, such as Fe, Al, Ca, Mg, and F, result in compounds other than $NH_4H_2PO_4$. The phosphorus availability of the impurity compounds was determined in 3 commercial MAP fertilizers produced from North Carolina, Florida, and Idaho phosphate rocks. Soluble compounds, including $NH_4H_2PO_4$, were washed out of the fertilizers, and the water-insoluble fraction was collected. The North Carolina, Florida, and Idaho MAP fertilizers contained 13, 16, and 17% water-insoluble fractions, respectively. Availability of phosphorus in each MAP fertilizer and water-insoluble fraction was determined in a greenhouse pot study using sorghum–sudangrass (*Sorghum bicolor*) as the test crop. There were no differences in dry matter weights or phosphorus uptake between reagent grade MAP and each MAP fertilizer. Lower dry matter weights and phosphorus uptake were produced from the water-insoluble North Carolina, Florida, and Idaho fractions (NC > FL > ID). Chemical analysis and characterization with optical microscopy, infrared spectroscopy, and X-ray diffractometry indicated 77.3, 73.8, and 73.6% $NH_4H_2PO_4$ in the North Carolina, Florida, and Idaho MAP fertilizers, respectively. $MgAl(NH_4)_2H(PO_4)_2F_2$, $AlNH_4HPO_4F_2$, and $FeNH_4(HPO_4)_2$ were identified as water-insoluble phosphorus compounds. There was a significant negative correlation between the percent phosphorus present as $MgAl(NH_4)_2H(PO_4)_2F_2$ and the phosphorus availability. Increased crystallinity of the various water-insoluble compounds also contributed to reduced phosphorus availability.

Because current MAP (mono-ammonium phosphate) fertilizers are produced from chemically heterogeneous phosphate rock, they contain a number of compounds, in addition to soluble MAP. The impurity compounds of MAP fertilizers are mostly amorphous and contain elements such as P, Al, Fe, Ca, Mg, and F (1). The impurities cause processing difficulties and result in fertilizer products with varied composition. The effect of the impurities on the agronomic availability of phosphorus has been determined in water-insoluble fractions of Australian fertilizers and other MAP and DAP (di-ammonium phosphate) fertilizers produced in 1980 (2). Lower plant dry weight and phosphorus uptake by corn were obtained from the water-insoluble fraction than from reagent grade $Ca(H_2PO_4)_2$. The predominant compounds in the water-insoluble fraction were amorphous Fe and Al phosphates.

The objectives of this present study were to determine the agronomic availability of phosphorus in the water-insoluble fraction of 3 commercial MAP fertilizers produced from North Carolina, Florida, and Idaho phosphate rocks and to identify the water-insoluble impurity compounds in these fertilizers by optical microscopy, infrared spectrophotometry, and X-ray diffractometry.

Experimental

Phosphorus Sources

Three commercial MAP fertilizers (11–52–0 grade) produced from North Carolina, Florida, and Idaho phosphate rocks were used in this present study. The water-insoluble MAP fraction was collected by mixing 200 g portions of MAP with 1 L water, stirring 1 h, and filtering. Water-insoluble precipitate was rinsed with acetone and dried.

The term, "water-insoluble," is used in a relative sense. Water-insoluble precipitate has some solubility in water but is less soluble than $NH_4H_2PO_4$ washed out of the fertilizer. North Carolina, Florida, and Idaho MAP fertilizers contained 13, 16, and 17% water-insoluble fractions, respectively. A Florida phosphate rock with low phosphorus availability was used as a phosphorus source for agronomic comparison. Reagent grade $NH_4H_2PO_4$ from Fisher Scientific was also used for agronomic comparison as a highly available phosphorus source.

Total N, Al, Fe, F, Ca, Mg, Si, SO_4-S, Na, K, total H_2O, and free H_2O were determined in phosphorus sources (3). Water-soluble phosphorus was determined by AOAC methods **2.040** and **2.043** (4). Citrate-insoluble phosphorus was determined by AOAC methods **2.044, 2.045(a)**, and **2.046(b)** (4). Chemical analyses of phosphorus sources are presented in Table 1.

Greenhouse Procedure

Mountview silt loam soil (*Typic paleudult*) was limed to pH 7.2 with 4:1 $CaCO_3$:$MgCO_3$ mixture and fertilized with 75 mg K/pot as K_2SO_4 (1.5 kg air-dried soil per pot). Micronutrients were supplied as sulfate and borate salt mixture at 7 mg Mg/pot, 7 mg Fe/pot, 5.5 mg Mn/pot, 6.5 mg Zn/pot, 1.8 mg Cu/pot, 0.8 mg B/pot, and 19 mg S/pot. At planting, N was equalized to 150 mg/pot with NH_4NO_3. Additional N (75 mg N/pot) as 4:1 $Ca(NO_3)_2$:$Mg(NO_3)_2$ solution was supplied after the first crop was harvested.

All phosphorus sources were pelleted with Carver press at 10 000 psi. Total MAP fertilizers and reagent grade $NH_4H_2PO_4$ were ground to powder before pelleting. After pelleting, materials were crushed and granules between 2 and 4 mm in diameter were collected. Phosphorus sources were applied to soil at application rates of 20, 40, and 60 mg phosphorus/kg soil. Additional phosphorus application rates of 100 and 180 mg phosphorus/kg soil were used with reagent grade MAP. Phosphorus sources were placed 5 cm below soil surface after top layer of soil was removed. Granules were placed at 2, 5, and 8 sites for phosphorus rates of 20, 40, and 60 mg phosphorus/kg soil, respectively. Granules were also placed at 8 sites at 100 and 180 mg phosphorus/kg soil. Granules were uniformly placed at specific sites by using 10.2 cm diameter Plexiglas circular template containing 1 cm diameter holes.

Eight sorghum–sudangrass (*Sorghum bicolor*; 'HW 6986') seeds were planted 2.5 cm deep in each pot on October 21, 1987, thinned to 5 plants after 1 week, and harvested on November 24, 1988, by cutting 4 cm above crown roots. Regrowth was harvested on January 5, 1988. Average daily maximum and minimum temperatures were 33 and 17°C for the first crop and 27 and 17°C for the second crop. Harvested plants were oven-dried, weighed, and ground in a Wiley mill

Presented at the Symposium on Fertilizers: Phosphate Evaluation and Analysis, 102nd AOAC Annual International Meeting, August 29–September 1, 1988, Palm Beach, FL.

Use of brand names does not constitute any endorsement of the instrumentation.

Table 1. Chemical analyses of the MAP fertilizers, water-insoluble fractions (WI-MAP), Florida phosphate rock (FL PR), and reagent grade $NH_4H_2PO_4$ (RG MAP)

Fertilizer	N, % w/w	P, % w/w	% P of total P		AOAC "available" P soluble in H_2O, %	Element content, % w/w										
			Water-soluble	Citrate-insoluble		Al	Fe	F	Ca	Mg	Si	SO_4-S	Na	K	Total H_2O	Free H_2O
NC[a] MAP	11.5	22.8	96.5	0.04	96.5	0.27	0.72	0.95	0.42	0.86	0.18	2.25	0.19	0.15	2.0	0.8
FL[b] MAP	11.5	22.7	92.5	0.04	92.5	0.78	1.52	0.98	0.33	0.45	0.09	1.92	0.12	0.29	1.7	1.0
ID[c] MAP	11.2	22.6	91.2	0.9	92.0	0.96	0.58	1.98	0.80	0.62	0.30	1.94	0.10	0.08	2.6	1.0
NC WI-MAP	6.3	16.8	38.2	5.5	40.4	1.97	5.45	5.98	1.29	3.93	1.97	0.43	0.18	0.20	13.9	3.7
FL WI-MAP	4.1	14.5	6.9	6.7	7.4	4.86	9.61	10.43	0.87	2.66	0.85	0.36	0.40	0.23	10.7	6.5
ID WI-MAP	3.9	14.0	4.4	14.8	5.2	5.46	3.43	10.97	2.57	3.48	3.35	0.16	0.08	0.07	12.7	5.6
FL PR	ND[†]	14.2	2.0	83.0	11.8	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
RG MAP	12.1	26.9	100	<0.04	—	<0.1	<0.1	0.02	<0.1	<0.1	0.02	<0.01	0.1	0.1	<0.1	<0.1

ND = Not determined.
[a] NC = North Carolina.
[b] FL = Florida.
[c] ID = Idaho.

for chemical analysis. Plant samples were digested by dry ashing 6 h at 475°C, dissolved in 2N HNO_3, and filtered. Filtrate was analyzed for phosphorus by the colorimetric method outlined by Murphy and Riley (5).

The greenhouse experiment had a split plot design with 4 replications. Phosphorus application rate was the main plot, and phosphorus source was the split plot. Data were analyzed by analysis of variance with phosphorus rate, phosphorus source, and phosphorus rate * phosphorus source interaction as main effects. Single degree of freedom contrasts were chosen within phosphorus source for comparing effects of phosphorus source on dependent variables of dry matter weight and phosphorus uptake.

Availability indices (AI) were calculated to compare dry matter weight and phosphorus uptake from fertilizer treatments with that from reagent grade $NH_4H_2PO_4$. Average dry matter weight and phosphorus uptake from each fertilizer source was divided by those with reagent grade $NH_4H_2PO_4$ at phosphorus application rates of 30, 60, and 90 mg phosphorus/pot.

Compound Distributions in Phosphorus Sources

Total MAP fertilizers and water-insoluble fractions were analyzed by Nikon Optophot polarized optical microscope, Digilab 14C Fourier transform infrared spectrophotometer, and Scintag PADV X-ray diffraction system. Infrared and X-ray analyses of synthetic compounds (6) were used to identify the compounds in MAP fertilizers.

Distribution of compounds in total MAP fertilizers was calculated from qualitative information on the type of compounds provided by spectroscopic analyses and from quantitative information on total elemental content. Assumptions were made on calculating the distribution of compounds in the water-insoluble fractions. One assumption was that all defined water-soluble compounds dissolved and that all defined water-insoluble compounds remained undissolved in the washing procedure. This assumption was not entirely correct because there were differences in calculated elemental concentrations from calculated compound distributions and actual chemical analyses of the water-insoluble fraction. The differences may have been due to incongruent dissolution of water-insoluble compounds when soluble $NH_4H_2PO_4$ was washed out of the fertilizer.

Water contents of water-insoluble fractions had to be accounted for in calculating compound distributions in water-insoluble fractions. Since water could have been lost and added to water-insoluble fractions during the washing procedure, an actual value for water content was difficult to obtain. To facilitate the calculation of compound distributions in water-insoluble fractions, a second assumption was made that total water found in MAP fertilizers was contained within water-insoluble fractions. This assumption appeared adequate because calculated water content was approximately the same as analytically determined water content.

A third assumption was made that compounds containing Na and K were negligible and, thus, were not considered.

Results

Sorghum–sudangrass reached maximum dry matter weight at 100 mg phosphorus/kg soil with reagent grade $NH_4H_2PO_4$ in both harvests (Figure 1). The phosphorus application rates of 20, 40, and 60 mg/kg soil resulted in increased plant dry weights and, thus, were adequate for evaluating the availability of phosphorus in the water-insoluble MAP fractions.

Dry matter weights with reagent grade $NH_4H_2PO_4$ and

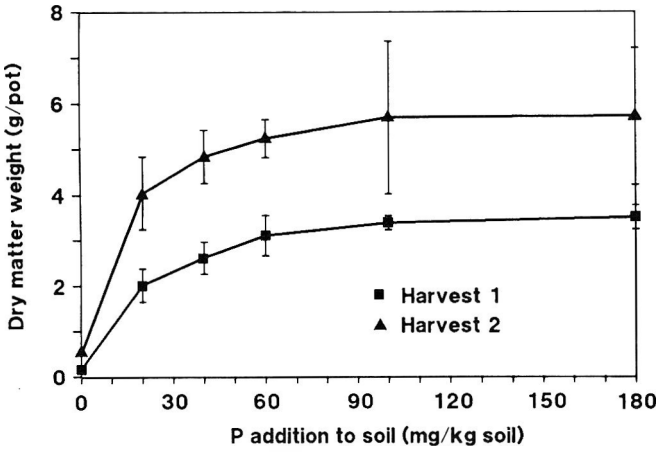

Figure 1. Yield response of sorghum-sudangrass to reagent grade $NH_4H_2PO_4$ in 2 harvests. Error bars represent standard deviation in the 4 replications.

Table 2. Analysis of variance for the dependent variables of dry matter weight and phosphorus uptake, and the independent variables of phosphorus rate and phosphorus source[a]

Source of variation	Harvest 1 Dry matter weight	Harvest 1 P uptake	Harvest 2 Dry matter weight	Harvest 2 P uptake
Rep	−	−	−	−
P rate	−	+	+	+
P source	+	+	+	+
P source contrasts: [b]				
P1 vs P3, P4, P5	− (−)	− (+)	−	− (+)
P2 vs P6, P7, P8	+ (+)	+ (+)	+	+ (+)
P3, P4, P5 vs P6, P7, P8	+ (+)	+ (+)	+	+ (+)
P3 vs P4, P5	− (−)	− (−)	−	− (−)
P4 vs P5	− (−)	− (−)	−	− (−)
P6 vs P7, P8	+ (−)	+ (+)	+	+ (−)
P7 vs P8	+ (+)	+ (+)	+	+ (−)
P rate × P source	+	+	−	+

[a] + and − denote significant and nonsignificant treatment effects, respectively, at p = 0.05. The + and − in parenthesis denote the significance of the interaction of P source contrast with P rate.

[b] P1 = reagent grade MAP, P2 = phosphate rock, P3 = NC MAP, P4 = FL MAP, P5 = ID MAP, P6 = NC water-insoluble-MAP, P7 = FL water-insoluble-MAP, and P8 = ID water-insoluble-MAP.

total MAP fertilizers in both harvests did not differ (Figure 2, Table 2). However, water-insoluble fractions produced lower dry weights than did total MAP fertilizers in both harvests. Lower dry matter weights were produced from the water-insoluble North Carolina, Florida, and Idaho fractions (NC > FL > ID). Availability indices calculated from dry weight for North Carolina, Florida, and Idaho water-insoluble fractions were 0.79, 0.58, and 0.36, respectively, in the first harvest and 0.79, 0.57, and 0.25 in the second harvest. The Florida phosphate rock produced lower dry matter weights than did the water-insoluble fractions.

The phosphate uptake followed similar patterns to plant yield (Figure 3 and Table 2). There were no differences in phosphorus uptake between reagent grade $NH_4H_2PO_4$ and the total MAP fertilizers. Availability indices calculated from phosphorus water-insoluble uptake for North Carolina, Florida, and Idaho fractions were 0.67, 0.40, and 0.13, respectively, in the first harvest and 0.87, 0.55, and 0.35, respectively, in the second harvest. The Florida phosphate rock produced lower phosphorus uptake than did the water-insoluble fractions.

Phases observed with optical microscopy in the MAP fertilizers and water-insoluble fractions are presented in Table 3, and X-ray diffraction patterns of the water-insoluble fractions are shown in Figure 4. Information provided by spectroscopic techniques and total chemical analyses indicated the total North Carolina, Florida, and Idaho MAP fertilizers contained 77.3, 73.8, and 73.6% $NH_4H_2PO_4$, respectively, and 10.2, 15.9, and 16% calculated water-insoluble compounds, respectively (Table 4). Phosphorus compounds observed in the water-insoluble fraction of each fertilizer were $Al(NH_4)HPO_4F_2$, $FeNH_4(HPO_4)_2$, and $MgAl(NH_4)_2H(PO_4)_2F_2$. The compounds $MgNH_4PO_4$ and $CaHPO_4$ were calculated in the water-insoluble North Carolina and Idaho fractions but were not detected by any spectroscopic analyses. Most phosphorus in the water-insoluble North Carolina and Florida fractions was contained in $FeNH_4(HPO_4)_2$ (Figure 5). Most phosphorus in the water-insoluble Idaho fraction was in the form of $MgAl(NH_4)_2H(PO_4)_2F_2$.

Discussion

The fact that dry matter weights and phosphorus uptake from reagent grade $NH_4H_2PO_4$ and MAP fertilizers were similar indicates the level of impurities in the fertilizers was not great enough to affect phosphorus availability and plant growth. The percentage of AOAC "available" phosphorus that was water soluble ranged from 92% for Idaho MAP fertilizer to 96.5% for North Carolina MAP fertilizer (Table 1). There is considerable evidence that a level of 60% water-

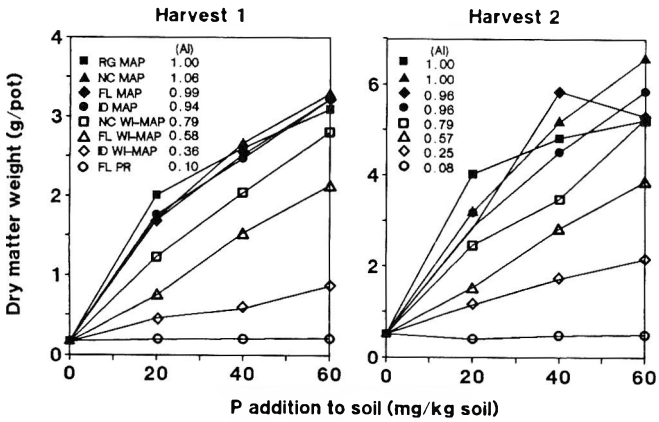

Figure 2. Dry matter weight of sorghum-sudangrass in 2 consecutive harvests as affected by 3 MAP sources, 3 water-insoluble fractions, reagent grade $NH_4H_2PO_4$ (RG MAP), and a Florida phosphate rock. AI = availability index.

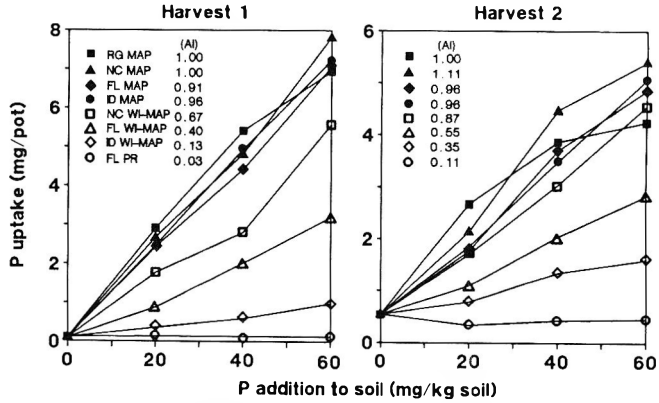

Figure 3. Phosphorus uptake of sorghum-sudangrass in 2 consecutive harvests as affected by 3 MAP sources, 3 water-insoluble MAP fractions, reagent grade $NH_4H_2PO_4$ (RG MAP), and a Florida phosphate rock. AI = availability index.

Table 3. Optical microscopy observations of MAP fertilizers and their water-insoluble fractions (WI-MAP)

	MAP
North Carolina	$NH_4H_2PO_4$ (70–80%) Optically amorphous material, n[a] = 1.510–1.530 (10–20%) Poorly crystallized material, n = 1.510–1.530 (<10%)
Florida	$NH_4H_2PO_4$ (70–80%) Optically amorphous material, n = 1.510–1.530 (10–20%) Poorly crystallized material, n = 1.510–1.530 (<10%) Mg-Al-F-xH_2O (<2%)
Idaho	$NH_4H_2PO_4$ (70–80%) Optically amorphous to cryptocrystalline material, n = 1.510–1.530 (10–20%) Mg-Al-F-xH_2O (<2%)
	WI-MAP
North Carolina	Optically amorphous material, n = 1.510–1.530 (99+%)
Florida	Optically amorphous material, n = 1.510–1.530 (95%) Mg-Al-F-xH_2O (5%)
Idaho	Optically amorphous material, n = 1.510–1.530 (50%) Crystalline material identified as $MgAl(NH_4)_2H(PO_4)_2F_2$ (30%) Crystalline material identified as $FeNH_4(HPO_4)_2$ (10%) Mg-Al-F-xH_2O (5%)

[a] n = Refractive index.

soluble phosphorus is adequate for maximum crop yields (7–9). More recently, there was no difference in phosphorus uptake or yield in field-grown pearl millet fertilized with 9 commercial MAP sources ranging from 81 to 96% AOAC "available" water-soluble phosphorus (Mullins, unpublished data).

The water-insoluble fractions of MAP fertilizer resulted in lower plant dry matter weight and phosphorus uptake than did the total MAP fertilizers. However, the water-insoluble

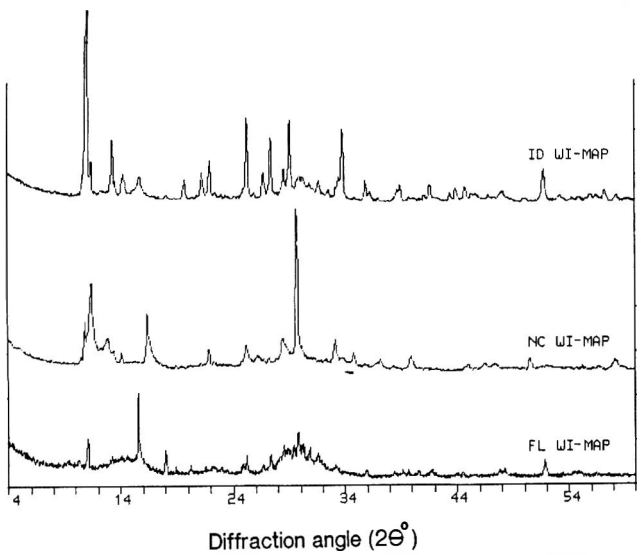

Figure 4. X-Ray diffraction patterns of the water-insoluble MAP fractions. The diffraction patterns all have the same intensity scale on the ordinate.

Table 4. Calculated percentage of compounds present in MAP fertilizers and their water-insoluble fractions (WI-MAP)

North Carolina				Florida				Idaho			
Compound	Water solubility[a]	MAP,[b] % w/w	WI-MAP,[b] % w/w	Compound	Water solubility[a]	MAP,[b] % w/w	WI-MAP,[b] % w/w	Compound	Water solubility[a]	MAP,[b] % w/w	WI-MAP,[b] % w/w
$MgAl(NH_4)_2H(PO_4)_2F_2$	–	1.6	15.7 c	$MgAl(NH_4)_2H(PO_4)_2F_2$	–	4.2	26.4 b	$MgAl(NH_4)_2H(PO_4)_2F_2$	–	6.5 b	40.6 abc
$FeNH_4(HPO_4)_2$	–	3.4	33.3 c	$FeNH_4(HPO_4)_2$	–	7.2	45.3 c	$FeNH_4(HPO_4)_2$	–	2.8 b	17.5 abc
$AlNH_4HPO_4F_2$	–	0.9	8.8 bc	$AlNH_4HPO_4F_2$	–	0.9	5.7 bc	$AlNH_4HPO_4F_2$	–	1.0	6.3 bc
$MgNH_4PO_4$	–	1.2	11.8	Mg-Al-F-xH_2O[c]	–	1.4 a	8.8 abc	$CaHPO_4$	–	0.9	5.6
MgF_2	–	0.4	3.9	$CaSO_4$	–	0.4	2.5	Mg-Al-F-xH_2O[c]	–	1.3 a	8.1 abc
CaF_2	–	0.3	2.9	SiO_2	–	0.1	0.6	CaF_2	–	0.2	1.3
$CaSO_4$	–	0.1	1.0	H_2O	–	1.7	10.7	$CaSO_4$	–	0.2	1.3
SiO_2	–	0.3	2.9	Total WI		15.9		SiO_2	–	0.5	3.1
H_2O	–	2.0	19.6	$Ca(NH_4)_2(SO_4)_2$	+	0.8		H_2O	–	2.6	16.3
Total WI		10.2		$(NH_4)_2SO_4$	+	6.1 b		Total WI		16.0	
$Ca(NH_4)_2(SO_4)_2$	+	1.6		$(NH_4)_2SiF_6$	+	0.2		$Ca(NH_4)_2(SO_4)_2$	+	2.5	
$Mg(NH_4)_2(SO_4)_2$	+	3.9		$(NH_4)_2HPO_4$	+	1.0		$(NH_4)_2SO_4$	+	2.5 b	
$(NH_4)_2SO_4$	+	2.6 b		$NH_4H_2PO_4$	+	73.8 abc		$(NH_4)_2SiF_6$	+	0.3	
$(NH_4)_2SiF_6$	+	0.3		Unknown		2.2		$(NH_4)_2H_3SO_4PO_4$	+	4.1	
$(NH_4)_2HPO_4$	+	1.9						$NH_4H_2PO_4$	+	73.6 abc	
$NH_4H_2PO_4$	+	77.3 abc						Unknown		1.0	
Unknown		2.2									

[a] Water-soluble compounds = +; water-insoluble compounds = –.

[b] Compounds identified by (a) optical microscopy, (b) x-ray diffraction, and (c) infrared spectrophotometry in the MAP fertilizer or water-insoluble MAP fraction (WI-MAP).

[c] Stoichiometry of Mg-Al-F-xH_2O may vary (12); $MgAl_2F_8 \cdot 2H_2O$ was used in the calculations.

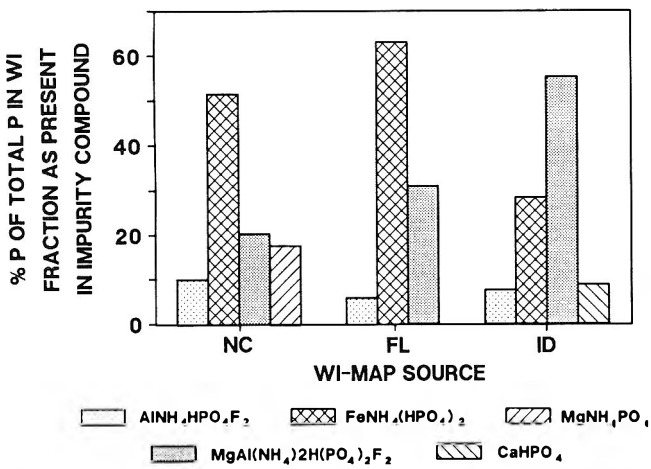

Figure 5. Phosphorus distribution in compounds present in water-insoluble MAP fractions. Values calculated from compound distributions shown in Table 4.

fractions did provide more available phosphorus for plant uptake than did the apatite in the Florida phosphate rock. The variation in phosphorus availability among the water-insoluble fractions was attributed to differences in phosphorus solubility and crystallinity of the compounds that comprise the water-insoluble fraction. There was a good correlation between the percent phosphorus, of total phosphorus, present in the water-insoluble fraction as $MgAl(NH_4)_2H(PO_4)_2F_2$ (Figure 5) and the availability indices calculated by dry matter weight (Figure 2) in both harvest 1 ($r = -0.98$) and harvest 2 ($r = -0.99$). The significant negative correlation may reflect low solubility and subsequent low availability of phosphorus in $MgAl(NH_4)_2H(PO_4)_2F_2$. Of several WI compounds found in MAP, synthesized $MgAl(NH_4)_2H(PO_4)_2F_2$ resulted in the highest citrate-insoluble phosphorus at 59% of total phosphorus (6).

Other compounds found in the water-insoluble fractions may also contribute to low phosphorus availability. Another major compound observed was $FeNH_4(HPO_4)_2$, which contained 28 to 63% of the total phosphorus in the water-insoluble fraction. Difficulty arises in contributing phosphorus availability solely to the percentage of phosphorus found in a particular compound because the degree of crystallinity of the individual compounds is an additional factor that contributes to decreased solubility. Dillard (6) found amorphous $AlNH_4(HPO_4)_2 \cdot nH_2O$ and $FeNH_4(HPO_4)_2 \cdot nH_2O$ to be totally soluble in neutral ammonium citrate, while crystalline forms of these compounds had 33 and 46% of total phosphorus as citrate insoluble. Taylor et al. (10) found amorphous Fe and Al compounds to be more available to plants than crystalline potassium iron phosphorite, potassium aluminum leucophosphite, and potassium iron leucophosphite. Colloidal iron phosphate resulted in greater dry matter weight of clover and ryegrass compared with crystalline strengite (11).

Although the compounds in the water-insoluble fractions were predominantly amorphous, optical microscopy indicated that the compounds in the water-insoluble Idaho fraction had a greater degree of crystallinity than those in the North Carolina and Florida fractions (Table 3). The spectra from X-ray diffraction (Figure 4) and infrared spectrophotometry (data not shown) showed several sharp peaks for the water-insoluble Idaho fraction, which also indicated a greater degree of crystallinity. In addition, the crystalline water-insoluble compounds were large enough (>200 μm) to be identified by X-ray diffraction in the total Idaho MAP fertilizer (Table 4). The crystalline nature of the water-insoluble Idaho compounds was believed to contribute to the low phosphorus availability of the water-insoluble Idaho fraction.

Two factors that controlled phosphorus availability of the compounds in the water-insoluble fractions were the type of compound and the degree of crystallinity of the compound. Each compound has an inherent solubility associated with it which controls the quantity of phosphorus released into soil solution. In addition, crystallinity of a compound controls solubility. If the type of compounds, degree of crystallinity of each individual compound, and solubility products were known, a valid prediction concerning the phosphorus availability in a water-insoluble fraction could be made.

The phosphorus fertilizer industry depends on the AOAC citrate solubility analysis to determine plant available phosphorus that can be reported in the fertilizer grade. The AOAC citrate analysis is empirical and was developed primarily for calcium phosphate fertilizers (7). With increased levels of metal impurities in phosphorus fertilizers, the citrate solubility test may not accurately determine plant available phosphorus. There were good negative correlations between availability indices (Figures 1 and 2) and citrate-insoluble phosphorus (Table 1) for the 3 water-insoluble fractions ($r = -0.95$ and -0.90 for availability indices from dry matter weight and phosphorus uptake, respectively, averaged from the 2 harvests). However, a study with 7 water-insoluble fractions, including the North Carolina, Florida, and Idaho water-insoluble fractions in this present study, showed discrepancies between availability indices and citrate-insoluble phosphorus for the North Carolina and Idaho sources (Mullins, unpublished data). In another study, agreement between plant growth and citrate-soluble phosphorus in water-insoluble fractions of DAP and MAP fertilizers was not good (2). In the future, a more accurate empirical analysis to determine phosphorus availability may be needed if the impurity levels in MAP fertilizers become prominent.

Acknowledgments

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Monitoring of Fertilizer Plant Effluents for Phosphorus, Sulfur, and Metals Using Inductively Coupled Plasma Emission Spectrometry

JIMMIE D. ARDIS and ALICE M. BAKER
Tennessee Valley Authority, National Fertilizer Development Center, Chemical Research, Muscle Shoals, AL 35660

At present, the U.S. Environmental Protection Agency (EPA) lists total P, F, and total suspended solids as effluent limitations for fertilizer manufacturing point source discharges. Other contaminants, including metals, may be added on a plant-by-plant permitting basis. EPA test method 200.7 lists 25 metals that may be determined by inductively coupled plasma (ICP) emission spectrometry as dissolved, suspended, or total elements in drinking and surface waters and domestic and industrial wastewaters. A method to measure a large number of these metals, as well as phosphorus and sulfur in fertilizer plant effluent water, by ICP spectrometry is described. The ICP spectrometer offers quick, sensitive, and accurate determinations for all species of phosphorus and sulfur. Total P can be determined regardless of the species present (lower oxides, ortho, poly, or organic), and sensitivity to phosphorus (0.08 μg/mL) is greater than for most other methods. Sensitivity to sulfur (0.1 μg/mL) also is greater than for most other methods and can be determined as total, sulfate, sulfide, or sulfite by controlling sample preparation. The present paper addresses only the measurement of total S because methodology has not been completed for other species.

The inductively coupled plasma optical emission spectrometer (ICP/OES or ICP) has proven to be a very versatile tool for analyzing liquid samples with a variety of matrixes. Solutions that range from aqueous or weakly acidic to those with high salt or dissolved solids content and many organics can be analyzed.

Of the 73 elements detectable by ICP spectrometry (Figure 1), this laboratory has analyzed about 50. These include alkali metals, hydride formers, and Hg, all of which are more sensitive to other methods than ICP spectrometry. However, where speed, freedom from interferences (chemical or spectral), or data handling (spectra, reports, etc.) are more important than sensitivity, the ICP spectrometer can be used.

The ICP spectrometer was purchased because of its ability to determine sulfur and its capability for determining phosphorus better than was possible with an old direct current plasma (DCP) spectrometer. Expectations have been exceeded, and the ICP spectrometer has become a workhorse for samples with low (<5%) to moderate (5–10%) amounts of P and S. An additional advantage is the ability to read P and S in the same solutions with metals.

Phosphorus is more sensitive to ICP analysis (0.08 μg/mL at 213.618 nm) than to most other methods. The Heteropoly Blue method is more sensitive, but has several disadvantages: (*1*) a complicated technique is required; (*2*) it is time consuming; (*3*) Fe, NO_3, Si, Ge, and As interfere; and (*4*) fuming is usually required to hydrolyze P and eliminate interferences. Total P is determined by the ICP spectrometer whether it is present as ortho, poly, organic, or a lower oxide. Table 1 lists typical instrument operating parameters.

The detection limit for S is 0.1 μg/mL at 180.731 nm, which also is better than for most other methods. Table 1 lists instrument parameters. Total S is measured if the sample is fully oxidized. Sulfide or sulfite may be measured by controlling the sample preparation. Work is continuing on a method for S species.

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Discussion of Methodology

Apparatus

A Perkin-Elmer Plasma II spectrometer with vacuum system and dual monochromators (monos) (Figure 2) is used. The "A" mono has a 3600 line grating and only scans wavelengths from 160 to 400 nm, with a resolution of 0.008 nm. The "B" mono has a 1800 line grating and scans wavelengths from 160 to 800 nm, with a resolution of 0.012 nm. This arrangement provides for better resolution on "A" and better spectral through-put on "B" where most elements are read. Even though this is a sequential instrument, read time is minimized by using both monos.

Sampling and Preparation

Samples studied included effluents from a phosphate mine, a research/pilot facility, and two fertilizer plants. Water from a fertilizer plant holding pond was selected as typical. Data from this sample were used to illustrate the results of a qualitative scan assay (Table 2) and a quantitative read (Table 3). Samples were taken and prepared by the U.S. Environmental Protection Agency (EPA) procedure (1) according to the type of data desired. For the determination of dissolved and suspended elements, the sample must be filtered through a 0.45 μm membrane filter as soon as practical after collection. For dissolved elements, the filtrate is acidified with HNO_3 (1 + 1) to pH 2 or less. For suspended elements, the filter plus suspended material from a measured volume is digested. For determination of total or total recoverable elements, the entire sample is acidified with HNO_3 (1 + 1) to pH 2 or less as soon as possible, preferably at the time of collection. Table 4, from EPA test method **200.7,** lists 25 elements to which this method is applicable. Also listed are recommended wavelengths and typical detection limits.

Instrumental Analysis

An effluent can be examined 3 ways, depending on the information desired:

(*1*) Qualitative scan: An assay of an unknown sample can be obtained as follows: An emission count of all detectable elements from the instrument files is obtained by calling all elements (select "periodic table") into "analyze" mode and by making one "sample" read without standardization. A similar read to deionized water and comparison of emission counts with those of the unknown will reveal significant contaminating elements (Table 2). A knowledge of element sensitivities and performance characteristics of the monochromators is needed for best interpretation of these data.

(*2*) Semiquantitative scan: An emission count of elements detected in the qualitative scan may be compared with the emission of elements in a mixed standard. This will approximate a quantity for each element.

(*3*) Quantitative read: A wavelength for each element to be read is selected from published tables (2–5); the emission intensity, detection limits, and potential spectral interferences are taken into consideration. Examples show resolved (Figure 3) and unresolved (Figure 4) peaks. A file is set up for each element to include the optimum instrument operat-

Li	Be											B	C	N			
Na	Mg											Al	Si	P	S		
K	Ca	Sc	Ti	V	Cr	Mn	Fe	Co	Ni	Cu	Zn	Ga	Ge	As	Se		
Rb	Sr	Y	Zr	Nb	Mo		Ru	Rh	Pd	Ag	Cd	In	Sn	Sb	Te	I	
Cs	Ba	La	Hf	Ta	W	Re	Os	Ir	Pt	Au	Hg	Tl	Pb	Bi			

Ce	Pr	Nd		Sm	Eu	Gd	Tb	Dy	Ho	Er	Tm	Yb	Lu
Th		U											

Figure 1. Periodic table that shows elements detectable by ICP spectrometry.

Table 1. Operating conditions of ICP spectrometer Perkin-Elmer Plasma II

	Phosphorus	Sulfur
Wavelength, nm	213.618 or 177.494 (for high Cu)	180.731
Viewing height, mm	10	6
PMT voltage, V	750	800
Sampling time, ms	200	200
Background correction	auto	auto
Sample rate, mL/min	1.0	1.0
Torch power, W	1250	1250
Ar flow, nebulizer, L/min	1.0	1.0
Ar flow, auxiliary, L/min	1.0	1.0
Ar flow, plasma, L/min	15.0	15.0
N_2 optics flush	on (below 200 nm)	on

ing parameters for that element as illustrated in Table 1. After the files are called into the "analyze" mode, the instrument is standardized against a mixed standard and an appropriate blank. After the concentrations are read, a comparison with the emissions of the qualitative scan gives an indication of the efficiency of the scan (Table 3). Very sensitive elements such as Ba, Be, and Sr show a significant emission but low concentration when analyzed quantitatively. Conversely, less sensitive P and S show a significant concentration even though scanning emission did not warrant listing them in Table 2.

Conclusions

ICP analysis provides (*1*) a method for quickly assaying an

Table 2. Effluent emissions vs blank

Element	Deionized water, blank Em	Fertilizer plant effluent water, effluent Em
Mg	25	2894
V	202	1936
Be	697	1213
Ca	137	39880
Al	285	567
Sr	268	2398
Ba	333	1510
Mn	9	25
Na	119	1578
K	14	18

Table 3. Emission vs effluent concentration

Element	Emission	Concn, μg/mL
Mg	2894	1.3
S	10	1.8
V	1936	0.8
Be	1213	<0.003
P	12	0.1
Ca	39880	6.8
Al	567	0.1
Sr	2398	<0.008
Ba	1510	0.03
Mn	25	0.17
Na	1578	2.2
K	18	0.4

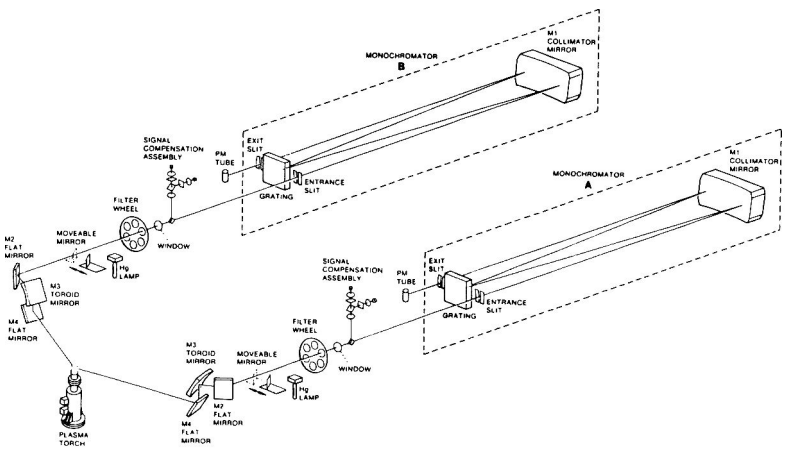

Figure 2. Schematic of dual monochromators.

Table 4. Recommended wavelengths and estimated instrumental detection limits

Element	Wavelength, nm	Estimated detection limit, μg/L
Aluminum	308.215	45
Arsenic	193.696	53
Antimony	206.833	32
Barium	455.403	2
Beryllium	313.042	0.3
Boron	249.773	5
Cadmium	226.502	4
Calcium	317.933	10
Chromium	267.716	7
Cobalt	228.616	7
Copper	324.754	6
Iron	259.940	7
Lead	220.353	42
Magnesium	279.079	30
Manganese	257.610	2
Molybdenum	202.030	8
Nickel	231.604	15
Potassium	766.491	—
Selenium	196.026	75
Silica (SiO_2)	288.158	58
Silver	328.068	7
Sodium	588.995	29
Thalium	190.864	40
Vanadium	292.402	8
Zinc	213.856	2

unknown solution (Table 2 shows that only 10 of 73 elements scanned give a significant emission increase over that of deionized water, and Table 3 shows Be and Sr to be below instrument detection limits even though they had a significant emission); (*2*) a tolerance for a variety of solution matrixes; (*3*) analysis with a minimum of sample preparation; (*4*) qualitative, semiquantitative, or quantitative analysis, as desired; and (*5*) analysis of S species.

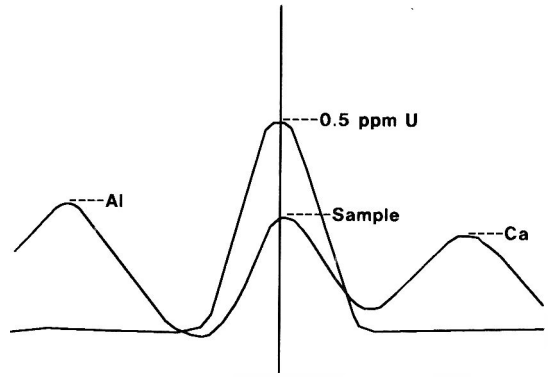

Figure 3. Example of resolved peaks, illustrated with spectra from ICP data files.

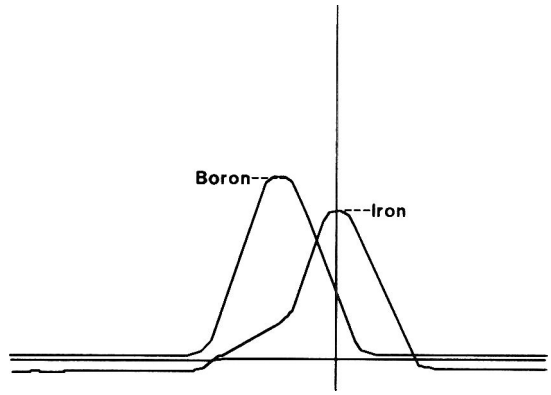

Figure 4. Example of unresolved peaks, illustrated with spectra from ICP data files.

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Comparison of AOAC and ISO Methods for Extraction of Water-Soluble Phosphates in Fertilizers

JAMES A. FARLEY
Tennessee Valley Authority, National Fertilizer Development Center, Chemical Research, Muscle Shoals, AL 35660

A wide range of phosphate fertilizers were analyzed for water-soluble phosphorus to compare 2 recognized methods: AOAC method 2.040 and the International Organization for Standardization (ISO) method No. 5316. In general, the ISO method extracted more phosphorus from triple and normal superphosphates; the AOAC method extracted more phosphorus from diammonium, monoammonium, and nitric phosphates. Both methods exhibited similar precision. The results indicate that care must be exercised when data for water-soluble phosphate are reported.

Over a century ago, AOAC was organized in the United States. The organizers met for the first time in 1884 to draft a constitution. In the first article, one of the stated objectives was "to secure, as far as possible, uniformity and accuracy in the methods and results of fertilizer analysis." At that meeting, a set of methods for the analysis of phosphorus was adopted, which served as a starting point for the methods we use today (1).

Changes have come slowly, and then only after considerable research. Today, the meaning of total phosphorus is clear enough, and water-soluble phosphorus usually means monoammonium phosphate (MAP), diammonium phosphate (DAP), or monocalcium phosphate (2). Available phosphorus is determined by using either the direct or indirect method, but both procedures use water extraction to remove water-soluble compounds before the citrate extraction. Because water solubility is not usually reported, its significance is masked by "availability."

In prior studies, Johnson and Farley (3) presented data to show that the quantity of water and technique of wash used to extract the water-soluble fraction from fertilizers significantly effect the result for available P_2O_5. This is most evident in triple superphosphate (TSP) fertilizers.

The International Organization for Standardization (ISO) was organized in the early 1970s and carries out its activities through technical committees with worldwide membership. The ISO working group that deals with methods development for phosphorus in fertilizer had its first meeting in 1973, and ISO method No. 5316 was developed and circulated to members by early 1976.

Because few data have been published on water-soluble methods, the present study was conducted to compare the official AOAC method **2.040** (4) with the more recently recognized ISO method No. 5316 (5). The 2 procedures are totally different, and there was reason to believe that one might give noticeably better precision than the other.

Experimental

Samples

To investigate the possible differences between the AOAC and ISO methods, 14 materials were chosen to represent the wide range of commonly produced phosphate fertilizers in the United States and Europe. A description of each sample, the source of phosphorus, and a value for available P_2O_5 content are listed in Table 1. Because these samples have been used in studies conducted previously by AOAC, The Fertilizer Institute (TFI), and ISO, they are well characterized. Samples 1–4 were TSPs, samples 5 and 6 were normal superphosphates (N-super), samples 7–10 were DAPs, and samples 11 and 12 were MAPs. Samples 13 and 14 were mixed fertilizers in which the phosphoric acid originated from nitric phosphate. Considerable data have been collected on these samples, but in areas other than water-soluble phosphorus.

Determinations

The 14 samples selected for study were analyzed in duplicate by the AOAC and ISO methods (Table 2). The procedures used to extract the water-soluble phosphorus can be summarized as follows: as specified in AOAC method **2.040,** 1 g of sample is weighed, placed on folded filter paper, and washed with active wash until 250 mL of filtrate is obtained. In ISO method No. 5316, 5 g of sample is weighed into a 500 mL flask, 400 mL water is added and the mixture is rotated 30 min, and then diluted to volume. From this point, the extracts were treated in the same manner: aliquots were hydrolyzed with nitric acid, and phosphorus was determined by the AOAC gravimetric method.

Results and Discussion

The experimental data were studied, and when interpreted, the results formed 2 distinct groups (Table 3). The first group consisted of the TSP and N-super samples. Precision was similar for the AOAC and ISO methods when the reproducibility of each method was compared; average variations were 0.17 and 0.19, respectively. The results also were compared to evaluate the amount of phosphorus extracted by each method. The AOAC method extracted less phosphorus than did the ISO method when applied to these materials.

The second group, composed of DAP, MAP, and nitric phosphates (Table 3), also showed similar precision when the reproducibility of each method was compared; average variations were 0.22 and 0.30, respectively. It was noted, however, that when the amount of phosphorus extracted by each method was compared, the AOAC method extracted significantly more phosphorus than did the ISO method when applied to this category of samples.

The results for sample 13 were excluded from the average variation because the low P_2O_5 values tended to amplify the variation, even though the range was similar to other samples. This unusual result should not be allowed to have an undue effect on the evaluation of the results.

Because sample 12 exhibited an unusually high difference between methods, the metals were determined by inductively coupled plasma (ICP) analysis. The values for iron and aluminum were >1 and >4%, respectively, indicating that some water-insoluble iron-aluminum phosphate compounds were present.

To settle the apparent difference in the amount of phosphorus extracted by each method, one TSP and one DAP sample were selected, each sample was extracted by the 2 methods, and pH of the solution from each sample was measured. The pH of the extraction is very important because

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Table 1. Samples selected for study to compare AOAC method 2.040 and ISO method No. 5316 for determination of water-soluble P_2O_5 in fertilizers

Sample No.	Sample description[a]	P_2O_5 source	Available P_2O_5, %[b]
1	TSP	Eastern U.S.	45.72
2	TSP	Morocco	47.58
3	TSP	Western U.S.	44.31
4	TSP	Eastern U.S.	46.62
5	N-Super	Eastern U.S.	19.60
6	N-Super	Portugal	20.22
7	DAP	Western U.S.	47.84
8	DAP	Eastern U.S.	46.83
9	DAP	Tunisia	45.96
10	DAP	Eastern U.S.	46.33
11	MAP	Eastern U.S.	52.37
12	MAP	Eastern U.S.	50.40
13	20-10-10	Nitric phosphate U.S.	10.18
14	15-15-15	Nitric phosphate Norway	14.73

[a] TSP = triple superphosphate; N-super = normal superphosphate; DAP = diammonium phosphate; MAP = monoammonium phosphate.
[b] Available by difference; AOAC gravimetric method used for total and citrate-soluble P_2O_5.

Table 2. Comparison of conditions in methods used to determine water-soluble P_2O_5 in fertilizers

Parameter	AOAC 2.040	ISO 5316
Sample size, g	1	5 (2.5)[a]
Volumetric flask, mL	not specified	500 (250)
Amount of water, mL	250	400 (200)
Time, min	60	30

[a] To facilitate use of the rotary shaker, the size of the sample, volumetric flask, and amount of water were divided in half.

free acid is released when water is added to a TSP or N-super sample, thus lowering the pH in the ISO method. The extraction then becomes a 30 min dilute acid extraction rather than a water extraction, as in the AOAC method. When water is added to DAP, MAP, and nitric phosphate samples, ammonia is released, which raises the pH in the ISO method. The extraction then becomes a 30 min dilute base extraction rather than a water extraction, as in the AOAC method.

Table 4. pH values of water extracts of triple superphosphate (TSP) and diammonium phosphate (DAP) fertilizers

	AOAC extract		ISO extract	
Washing No.	TSP	DAP	TSP	DAP
1	3.13	7.40		
2	3.50	7.30	3.30	7.40
3	4.76	7.06		
4	5.61	6.98		
5	5.97	6.93		
6	6.06	6.88		
7	6.12	6.84		
8	5.99	6.78		
9	6.20	6.85		
10	5.90	6.76		
11	6.18	6.84		
pH of distilled water = 5.87				

The AOAC method requires about 11 washings to obtain 250 mL of filtrate. The pH of each of the 11 washings from the TSP and DAP samples was measured; these values are shown in Table 4. The extracts of TSP and DAP from the ISO method gave pH values of 3.3 and 7.4, respectively.

It appeared from these tests that the free acid in the TSP and N-super samples influenced the results of the ISO method; higher values were yielded. In the DAP, MAP, and nitric phosphate samples, the reverse effect was observed.

In the AOAC method, the contribution by the free acid in the TSP and N-super and by ammonia in the DAP, MAP, and nitric phosphates was less apparent. After the third or fourth washing, the free acid did not contribute at all.

The method of extracting water-soluble phosphorus is significant to the final assay of fertilizer materials because the amount of available phosphorus is directly proportional to the amount of water-soluble phosphorus removed. The experimental data indicate that care must be exercised when water-soluble phosphorus results are reported because, according to the method used and the type of sample analyzed, the phosphorus may be acid or base soluble rather than water soluble due to the pH effects discussed earlier.

Table 3. Determination of water-soluble phosphorus in fertilizers by AOAC and ISO methods

Sample No.[a]	AOAC			ISO			Difference, AOAC − ISO
	P_2O_5, %[b]	Range	Variation[c]	P_2O_5, %[b]	Range	Variation[c]	
1	38.91	0.22	0.57	39.64	0.12	0.30	−0.73
2	44.91	0.04	0.09	45.12	0.18	0.40	−0.21
3	38.36	0.02	0.05	38.30	0.07	0.18	0.06
4	38.82	0.03	0.08	39.46	0.01	0.03	−0.64
5	17.00	0.04	0.24	17.29	0.02	0.12	−0.29
6	18.90	0.00	0.00	19.19	0.02	0.10	−0.29
Average variation			0.17			0.19	
7	43.64	0.11	0.25	43.09	0.18	0.42	0.55
8	41.78	0.11	0.26	41.28	0.03	0.07	0.50
9	44.18	0.00	0.00	43.70	0.20	0.46	0.48
10	42.00	0.03	0.07	41.27	0.14	0.34	0.73
11	47.86	0.02	0.04	47.18	0.05	0.11	0.68
12	40.02	0.05	0.12	39.00	0.19	0.49	1.02
13	1.96	0.06	3.06	1.64	0.02	1.22	0.32
14	11.25	0.09	0.80	11.15	0.02	0.18	0.10
Average variation[d]			0.22			0.30	

[a] See Table 1 for sample descriptions.
[b] Average of 2 independent analyses.
[c] Variation = (range/average) × 100.
[d] Excluding sample No. 13.

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Comparison of Traacs 800 Autoanalyzer with Official Methodology for Total and Available Phosphorus in Fertilizer

PETER F. KANE, ANNE L. ORCUTT, and JANET H. HUBER
Purdue University, Department of Biochemistry, West Lafayette, IN 47907

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A third generation of continuous flow analyzers, the Traacs 800, as configured for total P_2O_5 in fertilizers, was compared to the traditional gravimetric methodology. A t-test of routine samples gave t = −1.01 with 23 DF, indicating no significant difference at the 95% confidence level. The average bias, gravimetric minus analyzer, was −0.03% P_2O_5. The instrument configured for available P_2O_5 was also compared to second generation AAII instrumentation. Bias was shown to exist in both instruments, which could be overcome by use of matrix standards. When routine samples were extracted and compared on the 2 instruments using matrix standards, a t-test gave t = −1.54 with 26 DF, indicating no significant difference. The average bias, Traacs minus AAII, was −0.06% P_2O_5. Official AOAC method status will be pursued for both Traacs methodologies.

The Traacs 800 is a third generation continuous flow analyzer available from Bran and Luebbe, Technicon Industrial Systems, with a number of advances over second generation Auto Analyzer II (AAII) technology. The analyzer is completely integrated with a personal computer, which makes possible an intelligent sampler and a great deal of instrument flexibility. Sample "pecking" (introduction of air bubbles into the sample line), electronic debubbling of the flowcell, and reduced tubing diameters dramatically reduce carryover, allowing for rates of analysis typically over 100 samples/h. A reagent sequencer still to be developed will allow automatic instrument startup and shutdown. Some applications will make use of automatic dilution and resampling and automatic chemistry switching.

The Laboratory of the Indiana State Chemist has evaluated the instrument as configured for protein, phosphorus, and calcium in animal feeds and for total and available phosphorus in fertilizers. The protein methodology has been collaboratively studied (1) and recommended for adoption. The feed phosphorus and calcium papers are currently in preparation. The fertilizer total and available phosphorus methodologies are presented here, with the intention that collaborative studies be pursued.

Technicon currently supplies 2 fertilizer phosphorus manifolds. The "high precision" manifold 165-D007-01 (Figure 1) uses rock check standards to determine total phosphorus, while the "AOAC" manifold 165-D007-03 (Figure 2) is designed to parallel as closely as possible the AAII manifolds used in AOAC official methods for total and available phosphorus. The 2 Traacs manifolds are essentially identical except that the AOAC version incorporates heating coils for on-line digestion, and the sample pump tube sizes and flowcell pathlengths are adjusted to reflect differing standard concentration ranges. Technicon method 803-86T is supplied with the high precision manifold, and method 855-87T is supplied with the AOAC manifold.

Presented at the Symposium on Fertilizers: Phosphate Evaluation and Analysis, 102nd AOAC Annual International Meeting, August 29–September 1, 1988, Palm Beach, FL.

Total Phosphorus

Initial work compared the Traacs high precision method with the AOAC official gravimetric method for total phosphorus, **2.026** (2). Noteworthy differences between the Traacs "high precision" manifold and the current official automated manifold **2.032** (2) are listed below.

(a) Instead of a dilution loop for resampling back through the pump, the high precision manifold uses a dialysis membrane for dilution. This eliminates the least reliable part of the official manifold.

(b) Dilute $HClO_4$ used in the AOAC method was eliminated in the high precision manifold by initially mixing the sample with dilute HCl.

(c) A new phosphorus-compatible wetting agent, Steol, replaces Technicon's Agent A wetting agent.

(d) Vanadate and molybdate reagents are prepared separately and mixed on-line. Wetting agent is also injected on-line, making for much more stable reagents.

(e) H_2SO_4 replaces $HClO_4$ in the vanadate reagent.

(f) The standard range is much wider, 0.0032–1.6000 g P_2O_5/L instead of 0.150–0.350 g/L. Thus, the necessity to aliquot and dilute routine samples prior to autoanalyzer sampling is eliminated.

(g) The method as supplied by Technicon uses rock check samples as the standard material. Substituting NIST SRM 200, KH_2PO_4, allows for greater consistency with the official method.

The Traacs method specified a rate of 100 samples/h. The method precision was compared at 100 and 90 samples/h to determine if more precision could be obtained. At each of the sampling rates, 8 standards in the range 0.000–1.600 g P_2O_5/L were analyzed 9 times each in random order as if they were samples (Table 1). Calculated concentrations at both sampling rates showed good agreement with theoretical standard concentrations using a quadratic fit, but the standard deviations of the replicates appeared to be less on average at 90 samples/h. To be conservative, this rate was chosen for further work.

At 90 samples/h, preliminary analysis of routine samples indicated a negative bias on the Traacs instrument. Since the

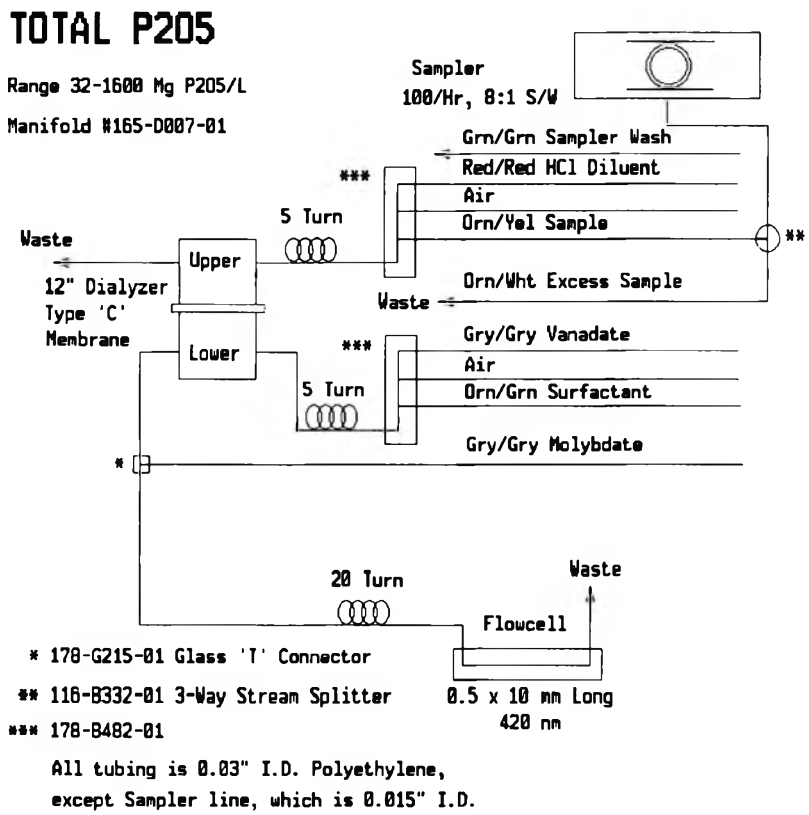

Figure 1. High precision P_2O_5 manifold.

standards were prepared as a water dilution, while the samples were prepared in an HCl–HNO_3 matrix, a matrix-effect test was designed. Three identical solutions of KH_2PO_4 were prepared, but with varying concentrations of HCl. The solutions were analyzed 12 times each (Table 2A). Since the differences in means were considerably less than the standard deviations, it was concluded there was no HCl matrix effect.

A similar test was designed to test for an HCl–HNO_3 effect. Solutions of KH_2PO_4 were prepared with and without HCl–HNO_3 (5 mL HCl and 30 mL HNO_3 per 500 mL) and each was analyzed repeatedly against water standards (Table 2B). The solution with HCl–HNO_3 showed a negative bias relative to the water standards.

Matrix standards were prepared by boiling 30 mL HNO_3 and 5 mL HCl for 30 min in a 500 mL volumetric flask,

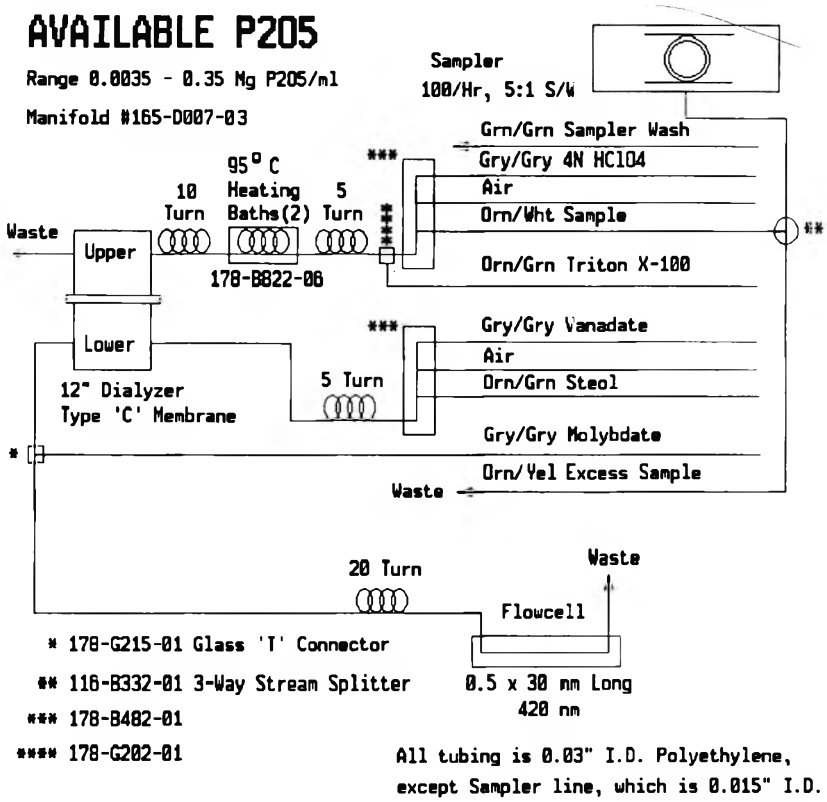

Figure 2. AOAC P_2O_5 manifold.

Table 1. Instrument precision, total phosphorus (g/L)

Theor. std curve	Calcd std Concn, av. of 9	Diff.	Std dev.
	At 100 samples/h		
1.6000	1.6000	0.0000	0.0046
1.3760	1.3752	0.0008	0.0055
1.1540	1.1492	0.0048	0.0027
0.9280	0.9269	0.0011	0.0025
0.7040	0.7026	0.0014	0.0023
0.4800	0.4783	0.0017	0.0024
0.2560	0.2571	0.0011	0.0015
0.0000	0.0025	0.0025	0.0026
	At 90 samples/h		
1.6000	1.6032	0.0032	0.0039
1.3760	1.3756	0.0004	0.0025
1.1540	1.1496	0.0045	0.0017
0.9280	0.9240	0.0040	0.0024
0.7040	0.7011	0.0029	0.0030
0.4800	0.4776	0.0024	0.0012
0.2560	0.2561	0.0001	0.0015
0.0000	0.0020	0.0020	0.0014

cooling, direct weighing NIST KH_2PO_4, and diluting to volume. Six standards in the range 0.000–1.200 g P_2O_5/L were prepared. This narrower range was still sufficient to handle 54% P_2O_5 samples without dilution, while improving the precision over the 0.000–1.600 g/L range. Eighteen 1 g samples of routine fertilizers, 3 KH_2PO_4, and 3 $NH_4H_2PO_4$ samples were digested with 30 mL HNO_3 and 5 mL HCl and diluted to 500 mL. The samples were analyzed once by the AOAC gravimetric method and 7 times over a period of 3 days by the Traacs method (Table 3).

The average bias, gravimetric minus Traacs, was -0.03% phosphorus. A t-test gave t = −1.01 with 23 DF, indicating no significant difference between the gravimetric and Traacs methods at the 95% confidence level. The standard deviations of the Traacs results are also given to provide an indication of the combined between-run and between-day precision to be expected from the instrument. The final methodology is summarized.

Total P_2O_5 in Fertilizer Semiautomated Method

Principle

Samples are extracted for total P_2O_5. Digested samples are reacted with vanadate and molybdate reagents, and the absorbance of the resulting complex at 420 nm is compared to that of standards in the range 0.000–1.200 g P_2O_5/L.

Table 2. Matrix effects, total phosphorus

A. HCl effect (n = 12)	KH_2PO_4 and 0 mL HCl/100 mL	KH_2PO_4 and 1 mL HCl/100 mL	KH_2PO_4 and 2 mL HCl/100 mL
Mean P_2O_5, g/L	1.2015	1.2020	1.2024
Std dev.	0.0039	0.0025	0.0035

B. HCl–HNO_3 effect (n = 6)	KH_2PO_4 and no HCl–HNO_3	KH_2PO_4 and 5 mL HCl–30 mL HNO_3/500 mL
Mean % P_2O_5	52.10	51.85
Std dev.	0.12	0.13

Table 3. Traacs total phosphorus with matrix standards vs AOAC gravimetric method

Sample	% P_2O_5, gravimetric result	% P_2O_5, mean, Traacs results	Diff.	Std dev.
$NH_4H_2PO_4$	61.86	61.80	0.06	0.14
	61.83	61.92	−0.09	0.21
	61.68	61.65	0.03	0.17
KH_2PO_4	52.11	52.23	−0.12	0.16
	52.07	52.20	−0.13	0.27
	52.00	52.05	−0.05	0.21
Routine samples	13.73	13.50	0.23	0.12
	6.27	6.69	−0.42	0.18
	15.07	15.57	−0.50	0.24
	47.89	47.79	0.10	0.20
	9.30	9.36	−0.06	0.15
	17.51	17.68	−0.17	0.11
	47.13	47.15	−0.02	0.19
	9.49	9.58	−0.09	0.13
	8.39	8.32	0.07	0.12
	46.65	46.72	−0.07	0.15
	6.73	6.62	0.11	0.16
	4.97	5.02	−0.05	0.11
	46.92	46.82	0.10	0.38
	9.81	9.81	0.00	0.21
	10.36	10.29	0.07	0.11
	32.37	32.25	0.12	0.15
	14.20	14.14	0.06	0.11
	9.02	8.97	0.05	0.06
		Av. bias = −0.03		

Apparatus

Bran & Luebbe Technicon Industrial Systems Traacs 800 continuous flow autoanalyzer with phosphorus manifold 165-D007-01, as illustrated in Figure 1.

Reagents

(a) *Concentrated wetting agent solution.*—Dilute 40 g Steol CS 460® (Brand-Nu Laboratories, Inc., Meriden, CT 06450) with 60 mL H_2O dropwise with vigorous mechanical stirring.

(b) *Sampler wash solution.*—Add 3 drops concentrated wetting agent solution **(a)** to 1 L H_2O and mix.

(c) *System wash solution.*—Dilute 5 mL HCl and 1 mL concentrated wetting agent solution **(a)** to 1 L with H_2O and mix.

(d) *Molybdate reagent.*—Dissolve 20.0 g ammonium molybdate in ca 800 mL H_2O. Adjust pH to 7.0 with NH_4OH, dilute to 1 L with H_2O, and mix.

(e) *Vanadate reagent.*—Add 35 mL H_2SO_4 to ca 700 mL H_2O. Add 0.150 g ammonium metavanadate, cool, dilute to 1 L with H_2O, and mix.

(f) *Sample diluent.*—Dilute 35 mL HCl to 1 L with H_2O, add 1 mL concentrated wetting agent solution **(a)**, and mix.

(g) *Surfactant solution.*—Dilute 4 mL concentrated wetting agent solution **(a)** to 1 L with H_2O and mix.

(h) *Phosphorus standard solutions.*—1.200, 0.960, 0.720, 0.480, 0.240, and 0.000 g P_2O_5/L. Prepare standards by adding 30 mL HNO_3 and 5 mL HCl to six 500 mL volumetric flasks and boil gently for 30 min. Accurately weigh to 4 decimal places KH_2PO_4 (NIST SRM 200 or equivalent) dried 2 h at 105°C, for the following target amounts of P_2O_5: 0.6000, 0.4000, 0.3600, 0.2400, 0.1200, and 0.0000 g, and dilute to volume with H_2O.

Determination

(a) Weigh 1 g sample into 500 mL volumetric flask, add 30

mL HNO_3 and 5 mL HCl, and boil until organic matter is destroyed; cool, dilute to volume, and mix.

(b) Analyze on autoanalyzer, Figure 1, using following parameters: 90 samples/h; 33/7 sample/wash ratio; use pecking; quadratic curve fit; base not in calibration; baseline drift correction; run protocol P, 3N, 6C, H, 2L, 30S, H, 2L, N, G, 2N, E; null peaks are high standard solution; gain references high standard in calibration sequence.

Note that sampling rate and sample-to-wash ratio are different from those recommended by Technicon. Also note that molybdate reagent is added 3–5 min after other reagents during startup, and removed 3–5 min before other reagents during shutdown. Rinse all lines ≥ 10 min with system wash solution before shutdown and rearrange dialyzer front inlet and waste lines so solutions do not syphon and allow membrane to dry out.

If standard concentrations are input into the Traacs in g/L:

$$\% P_2O_5 = (50)\ (\text{calculated peak conc.})/(\text{g sample wt})$$

Available Phosphorus

The AOAC manifold should be suitable for total as well as available phosphorus methodology. To date, this has not been verified, although as noted above, it is for all practical purposes identical to the high precision manifold. Noteworthy differences between the AOAC manifold as received and the AOAC official automated method for direct available phosphorus, **2.032**, are listed.

(a) Again, a dialysis membrane substitutes for the dilution loop. Along with eliminating this most troublesome part of the manifold, the membrane has the added advantage of blocking undigested citrate and colored molecules from the color development section of the manifold.

(b) Surfactant enters the $HClO_4$–sample mixture through its own pump tube, rather than being premixed with the acid, where it is unstable.

(c) Triton X-100® (Sigma Chemical Co., PO Box 14508, St. Louis, MO 63178) replaces Technicon's Wetting Agent A, but see further notes in the text.

(d) The vanadate and molybdate reagents are prepared separately as in the high precision manifold, and wetting agent (Steol) is again added separately.

(e) The residence time in the AAII heating bath is 9.15 min, while in the Traacs it is 8.65 min. This is not cause for concern, however, because hydrolysis of nonorthophosphates is not difficult. The critical factor in the AAII system was destruction of the citrate, and in the Traacs system the dialysis membrane blocks any undigested citrate, as mentioned in (a) above.

Unlike the high precision manifold, the AOAC manifold was received in a preliminary configuration, and had a significant stability problem, which was ultimately traced to 2 causes:

(1) The Triton X-100 wetting agent was found to cause drift. This surfactant was known to cause problems with the color development chemistry, but it was assumed that it would not diffuse through the membrane. Triton was also used because it does not break down in the presence of $HClO_4$ in the heating bath. When Triton was replaced with the same Steol wetting agent used in the color development portion of the manifold, there was significant reduction of drift. Steol also proved to be quite stable in $HClO_4$.

(2) Additional drift problems in the manifold were ultimately traced to the heat given off by the heating baths in the enclosed cabinetry. Unlike the AAII instrument, whose modules are located horizontally on the bench, the Traacs instrument is oriented vertically, which positions the heating baths directly underneath the colorimeter electronics. Also, the heating baths, located on the same metal plate on which the plumbing is mounted, warm the pins which hold the dialysis membrane blocks together. It was found that, if the instrument was operated with the front clear plastic manifold cover removed, and if the cabinetry doors were not opened and closed during operation, the instrument reached temperature stability in 15–20 min, the time it took to execute the startup procedures. With the temperature precautions and wetting agent change, the instrument became extremely stable.

The instrument was first checked for citrate effect. Two equimolar phosphate solutions were prepared, one without and one with ammonium citrate at the concentration found in samples. The solutions were analyzed on the Traacs 800 in alternating order (Table 4). The solution with citrate gave a lower response than the one without citrate. This was also confirmed by analysis of routine samples on the Traacs 800 using H_2O standards and by comparison with the same solutions analyzed by the AOAC official method, **2.032** (Table 5). The Traacs results were about 0.3% low relative to the AAII results. The bias was consistent across all samples.

In addition, repeated Traacs analysis of NIST KH_2PO_4 with citrate added averaged 51.84% P_2O_5, while analysis without citrate added averaged 52.13% P_2O_5, as compared to a theoretical value of 52.11% P_2O_5.

To further define the problem, 3 NIST KH_2PO_4 and 3 NIST $NH_4H_2PO_4$ samples were prepared with ammonium citrate solution, and analyzed 5 times with the same set of H_2O standards on both the Traacs and the AAII instruments (Table 6). It is evident that there is a negative Traacs bias, and also that there is a positive AAII bias. This bias problem was not surprising. It caused much difficulty when the original AAII automated method was developed (3). The remedy at that time was to attempt to balance the reagent concentrations in such a way as to compensate for the bias.

As an alternative, the current method uses matrix standards. This requires a wide standard concentration range to eliminate aliquoting, so that all samples have one level of ammonium citrate concentration, which then can be matched in the standard solutions. With appropriate computer correction, a wider range is now possible with AAII instrumentation (4). But utilization of the wide standard range is much facilitated with the Traacs software for curve fitting and raw data correction. Matrix standard deterioration is a problem, but standards can be preserved for about 1 month without clouding if kept in total darkness, or for longer periods if preserved with 2–3 mL chloroform.

A standard range of 0.000–1.200 g P_2O_5/L was chosen, the same range as used in the high precision manifold. This necessitated changing the flowcell path length from 30 to 10 mm. Also the amount of H_2SO_4 in the vanadate reagent was

Table 4. Available phosphorus citrate effect[a]

With citrate, g/L	Without citrate, g/L
0.2981	0.2987
0.2983	0.2997
0.2986	0.2999
0.2985	0.2993
0.2983	0.3001
Av. 0.2984	0.2995
SD 0.0002	0.0006

[a] Equimolar phosphate solutions prepared with and without ammonium citrate solution and analyzed alternately.

Table 5. Traacs available phosphorus method as supplied by Technicon vs AOAC automated method

Traacs results, %	AAII results, %	Traacs minus AAII
22.94	23.21	−0.27
23.02	23.49	−0.47
20.85	21.26	−0.41
31.44	32.05	−0.61
23.28	23.74	−0.46
12.80	13.13	−0.33
10.04	10.26	−0.22
23.37	23.74	−0.37
13.21	13.50	−0.29
20.89	21.26	−0.37
11.65	11.91	−0.26
13.25	13.52	−0.27
51.28	51.83	−0.55
20.44	20.67	−0.23
11.69	11.83	−0.14
27.22	27.55	−0.33
20.97	21.21	−0.24
24.58	24.87	−0.29
14.45	14.69	−0.24
23.24	23.53	−0.29
18.74	18.97	−0.23
19.68	19.98	−0.30
29.12	29.47	−0.35
10.12	10.29	−0.17
17.33	17.56	−0.23
9.67	9.85	−0.18
23.20	23.52	−0.32
22.70	23.00	−0.30
Av. 20.40	20.71	−0.31

changed from 15 to 35 mL, to match the reagent in the high precision manifold.

With these modifications, samples of NIST KH_2PO_4 and $NH_4H_2PO_4$ with ammonium citrate were prepared, and analyzed on the Traacs instrument using 0.000–1.200 g P_2O_5/L matrix standards, and on the AAII instrument using 0.150–0.350 g P_2O_5/L matrix standards (Table 7). The data from both instruments agree very precisely, and converge toward the correct theoretical values. The negative Traacs bias is eliminated. And the slight positive bias, that we suspect has always been present with the AAII instrument, has also disappeared.

Table 6. Available phosphorus bias, Traacs and AAII

NIST KH_2PO_4 52.11% P_2O_5		NIST $NH_4H_2PO_4$ 61.68% P_2O_5	
Traacs	AAII	Traacs	AAII
51.48%	52.55%	60.92%	62.97%
51.78	52.56	60.90	61.84
51.61	52.55	60.72	61.74
51.75	52.54	61.16	62.00
51.71	52.47	60.88	61.93
51.73	52.38	60.80	61.54
51.71	52.49	61.10	61.90
51.67	52.51	61.02	61.90
51.81	52.54	60.88	61.72
51.81	52.48	61.00	61.91
51.74	52.50	61.00	62.01
51.69	52.50	60.78	61.77
51.71	52.54	61.06	61.84
51.69	52.60	61.02	62.01
51.74	52.58	60.82	61.79
Av. 51.71	52.52	60.94	61.86

Table 7. Available phosphorus matrix standards used to analyze NIST materials

KH_2PO_4 52.11% P_2O_5		$NH_4H_2PO_4$ 61.68% P_2O_5	
AAII	Traacs	AAII	Traacs
51.94	52.00	61.61	61.56
51.97	51.97	61.59	61.56
52.07	52.01	61.57	61.52
Av. diff.	0.00		0.04

A group of routine samples was prepared and analyzed on the Traacs instrument with matrix standards, and on the AAII instrument with both matrix and water standards, Table 8. The results confirm the slightly higher AAII values when water standards are used. A t-test between the Traacs and AAII matrix results gave $t = -1.54$ with 26 DF, indicating no significant difference at the 95% confidence level. A final methodology for available phosphorus is summarized.

Direct Available P_2O_5 in Fertilizer Semiautomated Method

Principle

Samples are extracted for direct available P_2O_5. Extracted samples are mixed with 4N $HClO_4$ and digested at 95°C, and then mixed with vanadate and molybdate reagents; the absorbance of the resulting complex at 420 nm is compared to that of standards in the range 0.000–1.200 g P_2O_5/L.

Table 8. Available phosphorus, Traacs final configuration vs AAII, routine samples

AAII official result, %	AAII matrix result, %	Traacs matrix result, %
9.57	9.45	9.23
13.75	13.48	13.42
33.81	33.51	33.73
33.38	33.45	33.48
13.85	13.77	13.59
12.73	12.64	12.47
11.13	11.06	10.78
13.58	13.43	13.21
22.48	22.28	22.16
28.88	28.42	28.81
40.75	40.59	40.65
3.67	3.81	3.57
15.87	15.57	15.66
23.41	23.54	23.25
45.60	45.38	45.47
7.75	7.30	7.49
13.55	13.15	13.31
10.81	11.21	11.10
23.89	23.63	23.73
45.56	45.12	45.31
22.24	22.24	22.08
8.52	8.44	8.32
9.76	9.68	9.45
9.56	9.47	9.30
9.44	9.33	9.09
24.99	24.87	24.93
16.44	16.44	16.17
Av. 19.44	19.31	19.25

Average difference, Traacs − AAII Matrix = −0.06% phosphorus

Apparatus

Bran and Luebbe, Technicon Industrial Systems Traacs 800 continuous flow autoanalyzer with phosphorus manifold 165-D007-03, as illustrated in Figure 2. Note that the flow-cell pathlength is to be changed to 10 mm, and that Steol reagent is to be used in place of Triton X-100 reagent.

Reagents

High precision reagents (**a**), (**b**), (**c**), (**d**), (**e**), and (**g**) plus the following:

(**h**) *Perchloric acid 4N.*—Add 342 mL $HClO_4$ to ca 500 mL H_2O, cool, dilute to 1 L, and mix. Observe safety precautions in **51.028**, 14th Ed. (2), when handling perchloric acid.

(**i**) *Phosphorus standard solutions.*—1.200, 0.960, 0.720, 0.480, 0.240, and 0.000 g P_2O_5/L. Prepare standards by accurately weighing to 4 decimal places KH_2PO_4 (NIST SRM 200 or equivalent) dried 2 h at 105°C, to the following target amounts of P_2O_5: 0.6000, 0.4800, 0.3600, 0.2400, 0.1200, and 0.0000 g. Transfer to 500 mL volumetric flask, add 100 mL ammonium citrate solution (2.044) (2), dilute to volume, and mix. Store in total darkness or preserve with 2-3 mL $CHCl_3$.

Determination

(**a**) Weigh 1 g sample and extract as in **2.050(a)**.

(**b**) Analyze on autoanalyzer, Figure 2, using following parameters: 100 samples/h; 28/8 sample/wash ratio; no pecking; quadratic curve fit; base not in calibration; baseline drift correction; run protocol P, 3N, 6C, H, 2L, 30S, H, 2L, 4N, G, 4N, E; null peaks are high standard solution; gain references to high standard in calibration sequence.

Note that the sample-to-wash ratio is different than that recommended by Technicon. Also note that molybdate is added 15 min after all other reagents during startup, and removed 5 min before others on shutdown. Rinse all lines ≥ 15 min with system wash solution before shutdown, and rearrange dialyzer front inlet and waste lines so solution does not syphon and allow membrane to dry out.

If standard concentrations are input in g/L,

$$\% P_2O_5 = (\text{calculated sample conc./g SWT}) 50$$

Conclusions

Both the total and available methodologies agree well with AAII methodology. The direct available methodology on the Traacs instrument, with the refinement of the bias problem, represents a definite improvement in analytical quality over AAII instrumentation. Elimination of the necessity of sample dilution represents a reduction in labor requirements and the elimination of a source of error. Both methods are ready for collaboration.

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Availability of Phosphorus in Bone Meal

ALICE M. BAKER, JOE R. TRIMM, and FRANK J. SIKORA[1]
Tennessee Valley Authority, National Fertilizer Development Center, Chemical Research, Muscle Shoals, AL 35660

Two commercially available bone meal products (grades 0-12-0 and 6-12-0) were examined. Samples were prepared according to AOAC method 2.007, and total and available phosphorus contents were determined. Portions of these preparations were reground to pass through successively smaller sieves, and subsequent analyses indicated the availability of phosphorus to be directly proportional to fineness of grind. A quantity of the citrate–insoluble fraction of the bone meal was obtained by following AOAC extraction procedures. Agronomic studies were conducted that compared this insoluble fraction with the original bone meal material and with reagent grade $Ca(H_2PO_4)_2{\cdot}H_2O$. The data indicated poor correlation between the analytically defined and agronomically determined availability of phosphorus.

Interlaboratory imprecision of results for citrate-insoluble phosphorus has long been a problem. Fineness of grind has been shown to influence the neutral-ammonium citrate extraction of phosphate rock and triple superphosphate (TSP) (1, 2). A small, but consistent, increase in soluble phosphorus has been associated with a decrease in the particle dimension of various phosphate rocks. A study of factors that affect this extraction was conducted by Johnson and Farley (2). This examination of TSP showed a smaller percentage of citrate insolubility with a decrease in particle size. Their report cites fineness of grind as the most significant factor that contributes to the imprecision of results for citrate-insoluble phosphorus.

The present study was initiated to resolve discrepancies among laboratories in regard to the available phosphorus content of a degreased bone meal product. Familiarity with the previous study on phosphate rock and TSP led to the evaluation of particle size as a possible factor that contributes to the discrepancy of results for the bone meal material.

METHOD

See citrate-insoluble phosphorus: **2.044, 2.045(b), 2.046(b)** (3).

Discussion

A portion of commercially available 6-12-0 bone meal product was prepared according to AOAC method **2.007** (4). This method specifies that fertilizer materials and moist fertilizer mixtures should be ground to pass through a No. 20 sieve and that dry fertilizer mixtures, which tend to segregate, should be ground to pass through a No. 40 sieve. For the initial phosphorus determination, the material was ground to

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[1] Agricultural Research.

Table 1. Analysis of screen fractions of bone meal (6-12-0) ground to meet minimum AOAC specifications

Analysis	Screen fraction +40	−40 +60	−60 +100	−100 +140	−140 +200
% in fraction	60.0	18.3	10.6	9.1	1.9
% total P_2O_5	17.4	17.3	16.0	13.6	12.9
% NACI P_2O_5[a]	10.2	6.7	3.3	0.8	0.4
% available	7.2	10.6	12.7	12.8	12.5
% Ca	15.7	16.0	14.4	12.0	11.2
P:Ca	0.32	0.31	0.31	0.32	0.33

[a] Neutral-ammonium citrate insoluble phosphoric acid.

pass through a No. 20 sieve. Various screen fractions then were collected and analyzed to determine total and citrate-insoluble phosphorus and calcium contents. These data are presented in Table 1. Although the total phosphorus content differs slightly among the screen fractions, it varied in direct proportion to the calcium content, thus a constant phosphorus:calcium ratio was exhibited. On first examination, a more soluble phosphorus compound seems segregated in the smaller screen fractions; however, the consistency of the phosphorus:calcium ratio indicates that the phosphorus compound was the same throughout. Also notable was a significant decrease in the citrate-insoluble phosphorus content with a decrease in particle size.

A second portion of this product was prepared and then reground to pass through a series of successively smaller sieves. A sample was taken from each grinding for determination of total and citrate-insoluble phosphorus contents. The results of those determinations show that the available phosphorus content varied in inverse proportion to the particle size (Table 2). This procedure was repeated with a portion of commercially available 0-12-0 bone meal product. The results of this examination are shown in Table 3. Again, the particle size was found to significantly affect the phosphorus availability.

Although specifications for grinding are given in AOAC method **2.007,** they are only minimum requirements, and actual preparations may vary greatly among laboratories. For instance, some laboratories routinely grind all their materials to pass through a No. 40 sieve. If this procedure is followed, rather than the acceptable procedure of grinding to pass through a No. 20 sieve, our data show an increase in phosphorus availability of 21 and 30% for the 6-12-0 and 0-12-0 products, respectively. Because the market value of a phosphate fertilizer depends on its available phosphorus content, a small variation in grinding of these materials can have a significant financial impact on the manufacturer.

Greenhouse Studies

The current AOAC citrate extraction procedure was adopted in the 1930s. At that time, the method was modified so that analytical results for available phosphorus more nearly correlated with vegetative tests (5). To examine the validity of the AOAC extraction procedure in regard to agronomic availability of phosphorus in these materials, we initiated greenhouse studies. A quantity of the citrate-insoluble fraction of the 6-12-0 bone meal product was obtained by following AOAC extraction procedures **2.044** and **2.045(b).** This material (CIB) was compared with the original bone meal material (BON) and reagent grade $Ca(H_2PO_4)_2{\cdot}H_2O$ (REA) to determine relative availability to corn.

Table 2. Analysis of bone meal (6-12-0) ground to pass successively smaller screens

Analysis	Ground to pass mesh 20	40	100
% total P_2O_5	16.8	16.8	16.8
% NACI P_2O_5[a]	7.2	5.2	2.0
% available	9.6	11.6	14.8
Available as % of total	57	69	88

[a] Neutral-ammonium citrate insoluble phosphoric acid.

Table 3. Analysis of bone meal (0-12-0) ground to pass successively smaller screens

Analysis	Ground to pass mesh 20	40	100
% total P_2O_5	17.2	17.4	16.9
% NACI P_2O_5[a]	9.2	6.7	0.75
% available	8.0	10.7	16.2
Available as % of total	47	61	96

[a] Neutral-ammonium citrate insoluble phosphoric acid.

The original bone meal and the $Ca(H_2PO_4)_2{\cdot}H_2O$ were applied at 40, 80, 120, 240, and 480 mg phosphorus/pot. Because of a limited supply of the citrate-insoluble bone meal, it was not applied at the 240 and 480 mg levels. Three kg of Mountview silt loam soil was used per pot. The soil was limed to pH 6.4 with a 4:1 $CaCO_3$:$MgCO_3$ mixture and fertilized with 500 mg K/pot as K_2SO_4. Micronutrients were supplied in a mixture of sulfate and borate salts at rates of 14 mg Mg, 14 mg Fe, 11 mg Mn, 13 mg Zn, 3.5 mg Cu, 1.6 mg B, and 38 mg S per pot. The nitrogen fertilization rate was equalized to 600 mg/pot by using ammonium nitrate.

Corn was grown for 3 consecutive growth periods of 6 weeks each. At harvest, plants were cut 1 in. above the soil surface, oven dried, weighed, and ground for chemical analysis. Soil was collected after each harvest with a soil core. The soil was air dried and sieved through a 2 mm screen.

Plant samples were prepared by dry ashing, digested in 2N HNO_3, and filtered. Phosphorus was analyzed in the digested plant samples by the method of Murphy and Riley (6).

Corn dry matter weight increased with increased soil phosphorus application rates (Figure 1). At harvest 1, dry matter weights for BON were found to be significantly greater than those for CIB. The difference in dry matter weight between BON and CIB did not occur in the second and third harvests.

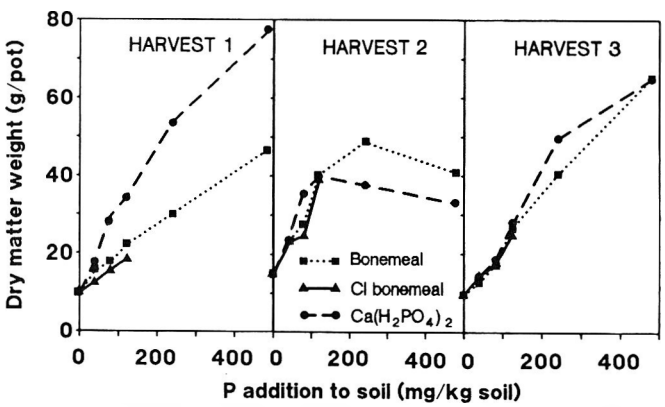

Figure 1. Yield of corn in 3 consecutive harvests as affected by 3 phosphorus sources.

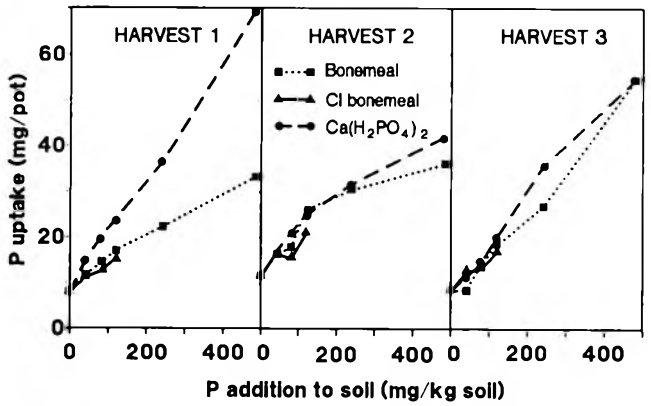

Figure 2. Phosphorus uptake of corn in 3 consecutive harvests as affected by 3 phosphorus sources.

As would be expected, phosphorus uptake followed a similar pattern to corn yield (Figure 2).

These data indicate that there is no substantial agronomic difference between the original 6-12-0 product and the citrate-insoluble fraction as applied to corn according to the parameters presented. Therefore, the AOAC method did not serve as a good predictor of phosphorus availability at the application rates tested.

Conclusions

The present study shows that the extractability of phosphorus by neutral-ammonium citrate is directly proportional to fineness of grind in 2 commercially available bone meal products. These analytical data indicate that consistent grinding of this material to pass through a 40 mesh sieve (as recommended for calcium metaphosphate) would increase precision among laboratories and more closely correlate with actual agronomic availability as indicated by the greenhouse studies.

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Mechanical Extraction of Available Phosphorus in Fertilizers

JOE R. TRIMM
Tennessee Valley Authority, National Fertilizer Development Center, Chemical Research, Muscle Shoals, AL 35660

A system is described for the extraction of available P_2O_5 in fertilizers by mechanically adding water and citrate to a glass cell that contains a ceramic filtering frit. Both water and citrate extractions are performed in the cell. The system can be operated manually or automatically. Samples tested included those from the Magruder and the Association of Florida Phosphate Chemists check sample programs for 1987. For all determinations, the values obtained with the mechanized system were within 1 standard deviation of the average AOAC value reported for each sample.

The current AOAC method for determination of available phosphorus in fertilizers (1) was developed in 1934. Except for a few changes, the method is the same today. The method was originally developed to differentiate among water-soluble phosphorus, reverted or citrate-soluble phosphorus, and unreacted phosphate rock. Greenhouse and field studies supported the assumption that the water-soluble and citrate-soluble phosphates were agronomically available.

Agricultural chemists have 2 primary concerns about the method. First, the method was developed in 1934 when the products tested were considerably different from today's products. Baker et al. (2) and Sikora (3) suggest that the method may not be applicable to all products. Second, because the method has remained essentially unchanged, there has been no opportunity to incorporate current laboratory technology.

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To change an empirical method such as the determination of available phosphorus, either of 2 criteria must be met: (*1*) the newly developed method would be accompanied by supporting greenhouse and field data showing that the availability found by the new method reflected the true agronomic availability; or (*2*) the new method, which might be a simplified, shortened, or automated method, would, for all practical purposes, give the same results as the official method. We chose the latter approach. The present paper describes a new technique for the mechanized extraction of available phosphorus.

METHOD

Reagents

(a) *Neutral ammonium citrate.*—Prepare as in **2.044** (1).

(b) *Filter aid.*—Celite (Manville, Mineral Panels Dept, Denver, CO 80217).

(c) *No. 2 paper pulp.*—Grind ca 5 g No. 2 filter paper in ca 500 mL water in blender.

(d) *5% Ammonium nitrate solution.*—Dissolve 50 g ammonium nitrate in water and dilute to 1 L.

Apparatus

(a) *Extraction cell.*—With body that can contain up to 250 mL; coarse frit at the bottom to serve as support for filter; and single outlet line (Figure 1).

(b) *Other equipment.*—Stirrer, peristaltic pump, and source of controlled heat.

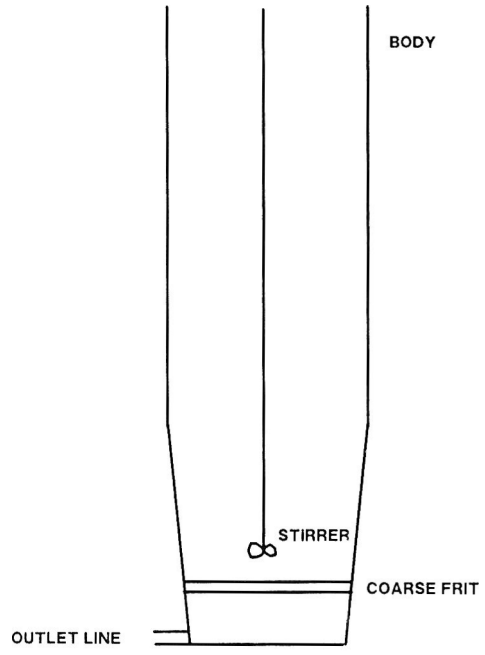

Figure 1. Extraction cell for available P_2O_5.

Preparation of Extract

Weigh 1 g sample into extraction cell. Add 0.5 g Celite, 25 mL No. 2 paper pulp, and 225 mL water. Stir 45 min. Discontinue stirring and let filtering medium settle ca 10 min. Use peristaltic pump to withdraw water-soluble fraction at ca 30 mL/min. With heated air or water, heat cell to 65°C. Pump 100 mL ammonium citrate, at 65°C, into cell. Portion of citrate must be pumped through frit to lift solids, and a portion must be directed to cell wall to wash down any adhering solids. Stir 1 h, discontinue stirring, and withdraw citrate extract in same manner as water extract. Wash residue 5 times with 15 mL portions of 5% ammonium nitrate solution. Combine water and citrate extracts for determination of available phosphorus.

Results and Discussion

The primary concern in developing the extraction cell was how to filter the water and citrate extracts. Nonpaper filtering mediums without filter aids were unsuccessful because of clogging, especially in samples that contained monoammonium phosphate (MAP). A combination of Celite, No. 2 paper pulp, and a coarse ceramic frit resulted in acceptable filtration of both the water and citrate extracts. During the water and citrate extractions, the Celite and paper pulp are suspended in the extracting liquid. When the stirring is discontinued, the paper pulp and Celite settle to the bottom of the cell. When vacuum is applied to the system, the paper pulp and Celite are compacted and form a thick pad that serves to filter the extracts and to yield a clear filtrate without clogging the system.

A single-step water extraction (225 mL water) is used. Although this technique contrasts with the multiple extractions currently used, repeated tests failed to show a significant difference between the 2 extraction techniques. These tests, performed on various samples of MAP, diammonium phosphate, triple superphosphate, and mixed fertilizer, compared a single-step water extraction (225 mL water) with the current AOAC water extraction.

The primary concern in the development of a method is the level of performance. For comparative results, samples from the Magruder and the Association of Florida Phosphate Chemists check sample programs for 1987 were analyzed using the extraction cell and the gravimetric quimociac method of analysis, **2.046 (a).**

Table 1. Available phosphorus determined in Magruder check samples

Sample No.	Material	AOAC av., %	Extraction cell, %
8701	Mixed fertilizer	8.14	8.12
8702	Triple superphosphate	46.56	46.72
8703	Mixed fertilizer	3.03	3.05
8704	Liquid fertilizer	21.03	21.03
8705	Mixed fertilizer	20.58	20.62
8706	Liquid fertilizer	11.85	11.84
8707	Diammonium phosphate	46.59	46.54
8708	Mixed fertilizer	13.03	13.01
8709	Liquid fertilizer	1.93	1.94
8710	Mixed fertilizer	15.33	15.29
8711	Mixed fertilizer	13.67	13.72
8712	Mixed fertilizer	2.37	2.35

The results (Tables 1 and 2) show that the new method performed well on check samples. The slight positive bias for available phosphorus is less than the average standard deviation.

Two samples that could not be properly analyzed with the current AOAC method were examined using the extraction cell. The first sample was a MAP with a citrate-insoluble value of over 1%. The AOAC method gave results ranging from 1.2 to 1.3%, but in every analysis, less than 250 mL water passed over the sample, regardless of the washing method. The sample presented no problem for the extraction cell and yielded results between 1.00 and 1.05% citrate-insoluble phosphorus, the lower results being attributed to the more efficient extraction of the water-soluble fraction. A second problem sample was a 10-34-0 fluid fertilizer that contained clay. With the AOAC method, less than 25 mL water passed over the sample, even with vacuum applied. In the extraction cell, the water and citrate extractions were performed without problems. Although the values for the citrate-insoluble phosphorus content of the sample could not be determined by the AOAC method, the value for citrate-insoluble phosphorus using the extraction cell was low (0.1%), as expected for fluid fertilizers.

Conclusions

The extraction cell described offers a new approach to the

Table 2. Available phosphorus determined in Association of Florida Phosphate Chemists (AFPC) check samples

Sample No.	Material[a]	AFPC av., %	Extraction cell, %
187	DAP	46.20	46.32
287	TSP	46.72	46.72
387	DAP	46.53	46.55
587	DAP	46.75	46.74
687	TSP	46.12	46.16
787	DAP	46.23	46.31
987	DAP	46.18	46.11
1087	TSP	46.18	46.40
1187	DAP	46.43	46.50
1287	TSP	46.32	46.19

[a] DAP = diammonium phosphate; TSP = triple superphosphate.

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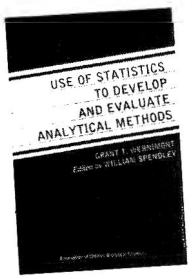

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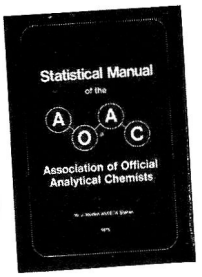

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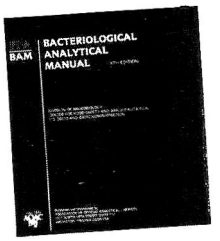

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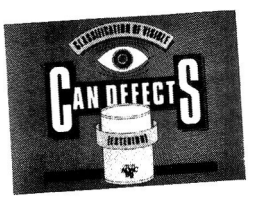

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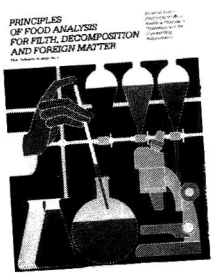

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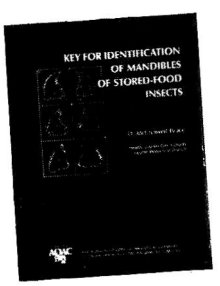

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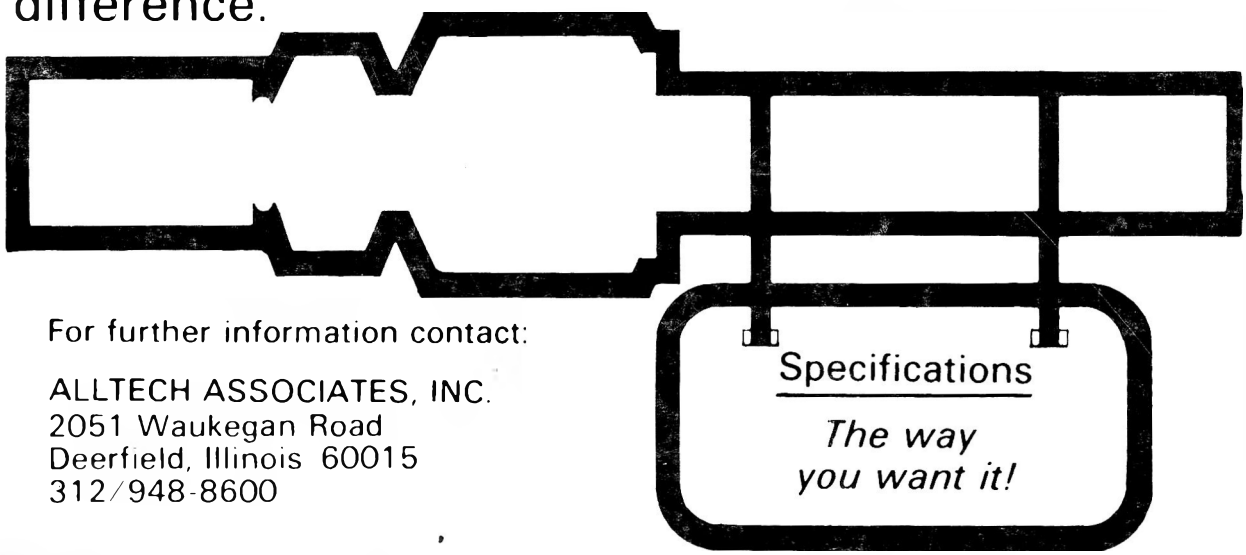

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