Association of Official Analytical Chemists

JOURNAL

Get the worst out of things.

UNITREX
Universal Trace Residue Extractor.

SGE's Universal Trace Residue Extractor – Unitrex – gets the worst out of your samples. Unitrex has been developed as an efficient and economical clean-up system for the recovery of pesticides and organic residues from meat fats, butter, cheese, vegetable oils and a wide range of samples.

Operating and maintenance costs have been kept to a minimum. Unitrex can process 10 samples simultaneously, and only small amounts of solvent and gas are required. The processing is fast and reliable – and Unitrex has rapid warm-up and $\pm 1°C$ temperature stability.

Performance is excellent. Recoveries for a range of pesticides, both organo-chlorine and organo-phosphorous compounds, are better than 90% for most samples at ppb levels, and the extract is clean enough for direct chromatographic injection into capillary columns or packed columns, even with highly sensitive detectors such as ECD, FDP. and NPD.

SGE

ASSOCIATION OF OFFICIAL ANALYTICAL CHEMISTS JOURNAL

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

JANUARY/FEBRUARY 1985
VOL. 68, NO. 1

ห้องสมุดกรมวิทยาศาสตร์บริการ

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NEW PRODUCTS

UV Detector

Model 116 high sensitivity, variable wavelength UV detector allows programming of all instrument parameters for routine and complex LC applications. Model 116 operates from 190 to 380 nm with a sensitivity range of 0.001–2.0 AUFS, monitors at 2 sensitivities simultaneously, and incorporates a built-in keypad and computer interface to set wavelength, cell pathlength, sensitivity, amplifier time constant, and fullscale output voltage. The optical compartment is purgeable, and the stepper motor-driven monochromator is laser-aligned for maximum precision. Contact: Gilson Medical Electronics, Box 27, 3000 W. Beltline, Middleton, WI 53562; 608/836-1551.
Circle No. 391

Drinking and Waste Water Analysis System

The Closed Loop Stripping Analysis (CLSA) system incorporates capillary GC and GC/MS, uses charcoal as an absorbent, uses small amounts of trapping material to minimize artifacts from filter bed materials, has high sensitivity in the 1–10 ng/L range, and has a 10 min extraction. Contact: Tom Jackson, Erba Instruments, 4 Doulton Pl, Peabody, MA 01960; 617/535-5986.
Circle No. 392

Filtration Apparatus

The Ultra-ware funnel/support assembly for micro filtration of liquids uses standard 47 mm diameter filter material. A TFE adapter which can connect to 1000 mL filter flasks, and provides total inertness. Funnel capacity is 300 mL (100 mL filter is optional). Contact: Kontes Scientific Glassware/Instruments, Spruce St, PO Box 729, Vineland, NJ 08360; 609/692-8500.
Circle No. 393

LC Column

Cyclobond I is based on the bonding of beta-cyclodextrin to a 5 μm silica gel to produce a non-nitrogen, non-hydrolytic chiral and general purpose phase. The method of separation is inclusion complexing, and Cyclobond I can separate enantiomers, positional isomers, and analogs in high aqueous solvent systems. Columns are 100 $\times$ 4.6 mm and 250 $\times$ 4.6 mm sizes. Contact: Advanced Separation Technologies Inc., 37 Leslie Ct, PO Box 297, Whippany, NJ 07981; 201/428-9080.
Circle No. 394

Liquid Chromatograph

The Series 1000 uses axial compression, high-efficiency columns (40 and 80 mm id) that are slurry-packed by the user for separation of pharmaceuticals, organic chemicals and intermediates, fragrance and flavor chemicals, essential oils, natural products, peptides, proteins, and polynucleotides. Efficiencies exceed 10 000 plates/m. A microcomputer with a menu-driven program in BASIC automatically controls operating parameters, including sample injection, fraction collection, recycling, column conditioning and backflush, and step gradients. Contact: Jack Cazes, Elf Aquitaine Development, PO Box 1678, Murray Hill Station,

Ion analyses in 20 seconds. Let us show you the way.

Flow Injection Analysis is the fastest and most effective method for routine analyses of ions in water, soil, fertilizers and beverages, for example analyses of NO_2^-, NO_3^-, PO_4^{3-}, NH_4^+, SO_2 and CO_2.

We have led the development of this method and it has resulted in the highly advanced system we have named FIAstar® System.

FIAstar provides for analyses at a very high speed, with production of results within 20 seconds. The consumption of both sample and reagents is extremely low and the rapid start-up/shut-down enable changes of method to be carried out very quickly. In addition FIAstar calculates the results immediately and shows them on a display and a printer/plotter.

Flow Injection Analysis is already well established in a number of fields, which have been the subject of hundreds of publications. Its growth and expansion into new fields is contin-

ual, thanks to intensive development of methodology in our application laboratories and in the hands of users all over the world.

Why not allow us to teach you about Flow Injection Analysis? We are world leaders in our fields and we hold seminars at frequent intervals as well as workshops in numbers of cities.

We will be happy to send you more information about the analytical technique of the future, Flow Injection Analysis.

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WE MAKE ROUTINE ANALYSIS SIMPLE.

A N N O U N C I N G

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

Quality Assurance Principles for Analytical Laboratories
by Frederick M. Garfield
former Assistant Administrator
U.S. Drug Enforcement Administration

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

The handbook also provides a rational and solidly based justification for commitment of resources to improved laboratory operation.

Drawing from published principles, practices, guidelines, and procedures, the book brings together the experiences of experts who have developed and implemented *successful* "QA" programs.

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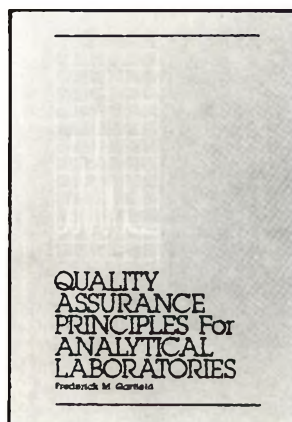
AOAC

To obtain book, send order and remittance to **AOAC**, 1111 N. 19th Street, Suite 210-J, Arlington, VA 22209 USA
(US funds only)

QUALITY ASSURANCE PRINCIPLES For ANALYTICAL LABORATORIES
Frederick M Garfield

NEW PRODUCTS

New York, NY 10156; 212/725-2555.
Circle No. 395

Reagent Dispenser
Oxford 5200 dispensers have 3 adjustable ranges: 0.2–0.8, 0.8–3, and 3–10 mL; thread diameters of 20–45 mm; vernier-type adjustment to yield volume settings with 1% accuracy and 0.5% precision; and construction of Fluoroplastic and glass, with ceramic ball valves. Contact: Sandra Osiecki, Monoject Scientific, Sherwood Medical, 1831 Olive St, St. Louis, MO 63103; 314/621-7788.
Circle No. 396

Desiccator Cabinet
The Auto Dry desiccator cabinet for chromatography plates, chemical standards, and general storage features a self-contained, heat-driven component that automatically absorbs moisture in the cabinet, and a 15 W incandescent light to provide additional heat for moisture absorption. The cabinet has 2 removable trays, one with slots for chromatography plates, and is made of 20 gauge, cold-rolled, epoxy-coated steel. Unit dimensions are $16 \times 12 \times 14$ in. and 25 lbs. Contact: Susan Gregory, Labconco Corp., 8811 Prospect, Kansas City, MO 64132; 816/333-8811.
Circle No. 397

Preparative Electrofocusing System
The Immobiline® gel has a pH gradient covalently bound to a polyacrylamide gel to allow preparative electrofocusing of large sample volumes and high protein loads, with high resolution and recovery. All procedural steps, from pouring the gel to recovering the sample, are detailed in Application Note 323, available free upon request. Contact: LKB Instruments, 9319 Gaither Rd, Gaithersburg, MD 20877; 301/963-3200.
Circle No. 398

Salt Analyzer
An automated analyzer simultaneously measures Na, K, and Cl content in any aqueous food extract and a wide variety of beverages by standard electrode methods. Up to 52 samples may be analyzed/h. A microprocessor allows complete automation, including periodic recalibrations. Contact: Project Manager, Nova Analytical, Nova Biomedical Corp.,

20 Ossipee Rd, Newton, MA 02164; 617/965-0800.
Circle No. 399

Database
The American Society of Hospital Pharmacists has produced a database, Drug Information Fulltext®, that represents the *American Hospital Formulary Service—Drug Information 84*® and the *Handbook on Injectable Drugs, Third Edition.* The database is available through Dialog Information Services, Inc., of Palo Alto, CA, and Bibliographic Retrieval Systems, of Latham, NY. The database represents more than 50 000 marketed drug products through generic, evaluative drug descriptions containing information on cautions, interactions, pharmacokinetics, chemistry, dosage, and stability. Contact: American Society of Hospital Pharmacists, 4630 Montgomery Ave, Bethesda, MD 20814; 301/657-3000.
Circle No. 400

TLC Developing Chamber
The Vega-T200X developing chamber has 2 solvent compartments, each of 15 mL capacity, permitting simultaneous development from either end of the TLC plate or sequential development; adjustable leveling legs to ensure linear solvent fronts; package of 25 wicks; and package of 5 saturation pads. Contact: Advanced Separation Technologies, Inc., 37 Leslie Ct, PO Box 297, Whippany, NJ 07981; 201/428-9080.
Circle No. 300

Liquid Injection System
The AS 550 Autosampler automatic liquid injection system for septumless, cold, on-column injectors and vaporizing capillary injectors avoids discrimination of sample components, eliminates many peak distortions, prevents thermal sample degradation, and avoids sample flashback. Features include injection of up to 60 samples, vaporizing injector stability, excellent volume reproducibility, full volume adjustability, accurate sample injection, and optional manual operation. Contact: Tom Jackson, Erba Instruments, 4 Doulton Pl, Peabody, MA 01960; 617/535-5986.
Circle No. 301

Solvent and Acid Cabinets

The Protector solvent storage cabinet safely stores solvents and other flammable liquids and features an automatically closing door in case of fire. The Protector acid cabinet is for storing and venting acids and other highly corrosive liquids and features a spill-stopping work surface. Cabinets are constructed of durable 18-gauge, dual-wall steel. Contact: Susan Gregory, Labconco Corp., 8811 Prospect, Kansas City, MO 64132; 816/333-8811.
Circle No. 302

Protein and Nucleic Acid Analysis System

Model 360 Mini Vertical Slab Cell allows analysis of protein and nucleic acid samples 2 to 3 times faster than 16 cm cells, with comparable resolution. Features include SDS-PAGE/silver stain analysis in 2.5 h: 8×8 cm gel: staining sensitivity to <1 ng; alumina-based heat transfer system; and optional casting stand for preparation of up to 10 gels. Contact: Bio-Rad Chemical Div., 2200 Wright Ave. Richmond, CA 94804; 415/234-4130.
Circle No. 303

Spectrophotometer Accessory

The Spectra-Scope, the latest in IR/FTIR accessories, speeds and simplifies the sampling and viewing with FTIR spectrophotometers by eliminating the use of external detectors and the need for cutting and preparation of samples. The Spectra-Scope also features image masking, micro positioning, interchangeable reflecting objectives, flexible illumination, and laser block and purge. Contact: Don Sting, Spectra-Tech, Inc., 652 Glenbrook Rd, Bldg No. 8, Stamford, CT 06906; 800/243-9186.
Circle No. 304

Photo Micron Sizer

Gilson Model SA-2000S features a larger CRT display and printer output in a new, larger, more detailed format. System accuracy is ± 1% over a range of 0.1–500 μm, with an analytical time of ≤ 30 min. The sizer operates at 0.05% solids concentration, with very good precision and repeatability. Contact: Gilson Company, Inc., PO Box 677, Worthington, OH 43085.
Circle No. 305

Repetitive Pipetter

The Stat-Matic II is a hand-held, compact, repetitive pipetter, with a total length of only 8 in. (20 cm). Because of its construction, the Stat-Matic II is inert to most common chemicals. Its light, smooth plunger action will not fatigue a user even over long runs. Contact: Glen Coultrip, Tri-Continent Scientific, Inc., 12541 Loma Rica Dr. Grass Valley, CA 95945; 916/273-8888.
Circle No. 306

Laboratory Hood

The Protector Series hood with integral motor/blower costs less than buying a hood and blower separately and is also easier and less expensive to install. Because the blower is not exposed to the elements, the hood requires less maintenance. Integral motor/blower hoods are quieter than most home appliances. Hoods are available in 4, 5, and 6 ft sizes. Contact: Susan Gregory, Labconco Corp., 8811 Prospect, Kansas City, MO 64132; 816/333-8811.
Circle No. 307

Test Kit for Mutagenic Substances

The S.O.S. Chromotest Kit is a rapid, colorimetric test for the detection of mutagenic substances. The kit can provide quantitative results in < 8 h with sensitivity equal to that of the Ames test. The kit can also detect toxic substances that usually create a false result in the Ames test. Each kit contains the necessary reagents and S-9 liver extract for activation of promutagenic substances. The S.O.S. Chromotest can provide up to 72 point determinations. Contact: Sally Lenhart, Gilford Instrument Laboratories, 216/774-1041.
Circle No. 308

Roller-Bottle Apparatus

The Wheaton Mid-Size Roller Apparatus fits larger bench top incubators and floor model incubators too small to accommodate the Wheaton Modular Roller Apparatus. This mid-size roller holds a total of 10 roller bottles ranging in size from 225 to 260 mm.

provides increased traction for bottles, facilitates the use of lightweight plastic bottles, and operates at speeds of 0.2–2.5 rpm, ensuring uniformity of speed. One additional deck can be added to increase the capacity to 15 bottles. Electrical requirements are: 105/120 VAC, 220/240 VAC, 50/60 Hz, 50 W. Contact: Beth Chain, Wheaton Instruments, 1301 North 10th St, Millville, NJ 08332; 609/825-1400 (Ext. 2237).
Circle No. 309

Metering Pump
The MiniPump VS is a low cost, variable speed metering pump featuring a new multi-piece liquid end which uses check valve cartridges for increased reliability and ease of maintenance, and an electronically controlled flow rate ranging from 0.5 to 5.0 mL/min for accurate and precise flow delivery. The MiniPump VS is pressure rated to 3000 psi. Contact: LDC/Milton Roy, PO Box 10235, Riviera Beach, FL 33404; 800/327-6182.
Circle No. 310

Literature

ASTM Standards Book
The 1984 Annual Book of ASTM Standards is now available. The book contains more than 7000 standards published in 66 volumes. Sixteen sections divide these volumes into related subject areas. For ordering information, contact: ASTM, 1916 Race St, Philadelphia, PA 19103; 215/229-5585.
Circle No. 311

New Products Catalog Supplement
Bulletin 484 features more than 50 new products, including micro filtration glassware, gas sampling tubes, EDB testing apparatus, and others. For a free copy, contact: Kontes, PO Box 729, Vineland, NJ 08360; 609/692-8500.
Circle No. 312

ANSI Standards
The 1984 edition of the Catalog of American National Standards lists all current ANSI-approved standards (about 8000). Institutional members and libraries that serve the general public receive the catalog and its supplements free. Supplements are issued several times a year. Nonmembers may obtain a copy of the catalog from ANSI Sales Department for $10.00. For more information, contact: Deborah Maskin, ANSI, 1430 Broadway, New York, NY 10018; 212/354-3315.
Circle No. 313

Applications Report
ESA, Inc. has published an applications report that details the sample preparation and analysis of various phenolic preservatives used in the food and cosmetic industries. Contact: ESA, Inc., 45 Wiggens Ave, Bedford, MA 01730; 617/275-0100.
Circle No. 314

Microsphere Bibliography
Over 742 citations covering the fundamental applications for regional blood flow using inert and biodegradable microspheres, plus many nonradioactive extensions of the technique are featured in the Microsphere Bibliography. Contact: Dick Reid, LKB Instruments Inc., 9319 Gaither Road, Gaithersburg, MD 20877; 800/638-6692 Ext. 282.
Circle No. 315

BOOKS IN BRIEF

Moisture Sorption: Practical Aspects of Isotherm Measurement and Use. By T. P. Labuza. Published by American Association of Cereal Chemists (AACC), 3340 Pilot Knob Rd, St. Paul, MN 55121, 1984. 150 pp. Price: $22.50 (AACC members)/$28.50 (nonmembers). ISBN 0-913250-34-1.

This water activity workbook provides mathematical examples and methods of predicting shelf life and moisture pickup of most baked, extruded, and dehydrated cereal-based products. Topics include typical sorption isotherms, use of the moisture sorption isotherm, and drawing the isotherm.

Cereal Polysaccharides in Technology and Nutrition. Published by American Association of Cereal Chemists, 3340 Pilot Knob Rd, St. Paul, MN 55121, 1984. 184 pp. Price: $18.00 (AACC members)/$23.00 (nonmembers). ISBN 0-913250-36-8.

This book discusses various food and industrial applications of many diverse cereal polysaccharides. It contains the latest information on cereal polysaccharide research, and elucidates the role of these carbohydrates in technology and health.

Advances in Pesticide Formulation Technology. By H. B. Scher. Published by American Chemical Society (ACS), 1155 Sixteenth St, NW, Washington, DC, 1984. 250 pp. Price: $44.95 (U.S. and Canada)/$53.95 (export). ISBN 0-8512-0838-7.

ACS Symposium Series No. 254, based on a symposium sponsored by the ACS Division of Pesticide Chemistry at the ACS 186th meeting, August 28–September 2, 1983, Washington, DC, treats new and wide-ranging developments in formulation techniques and technology, with emphasis on physical and chemical principles relating to pesticide dispersions (flowable formulations), controlled release formulations, computer applications, and surfactant utilization.

Drugs and Nutrients: The Interactive Effects. Edited by D. A. Roe and T. C. Campbell. Published by Marcel Dekker, Inc., 270 Madison Ave, New York, NY 10016, 1984. 624 pp. Price: $99.75 (20% higher outside U.S. and Canada). ISBN 0-8247-7054-4.

Volume 21 of the series Drugs and Pharmaceutical Science emphasizes the effects of nutrition on the disposition of foreign compounds and the effects of drugs on nutrition, while including the most recent advances, methodologies, and research goals in this field.

Food Constituents and Food Residues: Their Chromatographic Determination. Edited by J. F. Lawrence. Published by Marcel Dekker, Inc., 270 Madison Ave, New York, NY 10016, 1984. 632 pp. Price: $99.50 (20% higher outside U.S. and Canada). ISBN 0-8247-7076-5.

Volume 11 of the Food Science and Technology Series evaluates a wide range of chromatographic techniques for detection of important organic food constituents and residues of additives and contaminants at concentrations varying from trace levels to percent compositions.

Handbook of Vitamins: Nutritional, Biochemical, and Clinical Aspects. Edited by L. J. Machlin. Published by Marcel Dekker, Inc., 270 Madison Ave, New York, NY 10016, 1984. 632 pp. Price $79.50 (U.S. and Canada)/$95.25 (elsewhere). ISBN 0-8247-7051-X.

For each of the vitamins, Volume 13 of the Food Science and Nutrition Series details the chemistry, availability, and content in food; methods of bioassay and chemical analysis; metabolism; function; history; methods for evaluating overt or marginal deficiencies; nutritional requirements; interaction of vitamins with environmental factors, other nutrients, drugs, alcohol, and smoking; and the efficacy and hazards of their use at high dosages.

Proceedings of Seminar on Challenges to Contemporary Dairy Analytical Techniques. Distributed by Royal Society of Chemistry Distribution Center, Blackhorse Rd, Letchworth, Herts SG6 1HN, England, 1984. 337 pp. Price: 12£ (sterling).

The seminar was organized jointly by the International Dairy Federation, the Federation of European Chemical Societies, and the Association of Official Analytical Chemists and was concerned with the application of methods in quality control of dairy products and with

the techniques that will be in regular use in quality control.

Computers in Flavor and Fragrance Research. Edited by C. B. Warren and J. P. Walradt. Published by the American Chemical Society, 1155 16th St, NW, Washington, DC 20036, 1984. About 164 pp. Price: $29.95 (U.S. and Canada)/$39.95 (export). ISBN 0-8412-0861-1.

ACS Symposium Series No. 261, based on a symposium sponsored by the Division of Agricultural and Food Chemistry at the ACS 186th meeting, August 28–September 2, 1983, Washington, DC, explores the use of microcomputers in flavor and fragrance chemistry and in information management. Topics are presented by researchers in the field and include laboratory robotics and computer design of molecules.

Reference Edition Volume 2 1983. Edited by P. T. Shepherd. Published by Elsevier Science Publishers, PO Box 211, 1000 AE Amsterdam, The Netherlands, 1984. Also available from Elsevier Science Publishing Co., Inc., PO Box 1663, Grand Central Station, New York, NY 10163. 298 pp. Price: $92.25 (U.S. and Canada)/Dfl. 240.00 (elsewhere). ISBN 0-444-42303-6.

This second compendium of Trends in Analytical Chemistry (TrAC) contains the archival material published in TrAC in 1983. Articles include: Interface and Computer Corner, focusing on practical applications of computers and statistical methods in analytical laboratories; and Biotechnology Focus, highlighting the impact of biotechnology on analytical chemistry and analytical problems in biotechnological processes.

Evaluation of Analytical Methods in Biological Systems. Edited by R. A. De Zeeuw. Published by Elsevier Science Publishers, PO Box 211, 1000 AE Amsterdam, The Netherlands, 1984. Also available from Elsevier Publishing Co., Inc., Grand Central Station, New York, NY 10163. 338 pp. Price: $73.00 (U.S. and Canada)/Dfl. 190.00 (elsewhere). ISBN 0-444-42207-2.

Part B: Hazardous Metals in Human Toxicology reviews currently available techniques for the bioanalysis of metals and considers factors such as sample pretreatment, digestion techniques, recoveries and losses, sample throughput, cost, and automation. The volume also deals with toxicokinetics and dynamics of metals, distribution and elimination, biological changes, and symptomatology.

Modern Methods of Food Analysis. Edited by K. K. Stewart and J. R. Whitaker. Published by AVI Publishing Co., 250 Post Rd E, PO Box 831, Westport, CT 06881, 1984. 406 pp. Price: $49.50. ISBN 0-87055-X.

Based on the Seventh Basic Symposium of the Institute of Food Technologists, this volume by 21 authors in the fields of biochemistry, chemistry, food science, food technology, and nutrition provides contributions to the study and practice of food analysis, including computerization, sensory analysis, sample preparation, and automation.

Veterinary Pharmacology and Toxicology. Edited by Y. Ruckebusch, P. L. Toutain, and G. D. Koritz. Published by AVI Publishing Co., 250 Post Rd E, PO Box 831, Westport, CT 06881, 1984. 838 pp. Price: $56.00. ISBN 0-87055-442-5.

Based on the proceedings of the Second European Association for Veterinary Pharmacology and Toxicology, this volume provides current research in veterinary perinatal and comparative pharmacology and in pharmacological methods and technology, with emphasis on ruminant pharmacology and animal models. The assessment of carcinogenic properties of veterinary drugs, clinical utility of pharmacokinetics, and neonatal treatment of diseases in calves are also discussed. New drugs such as detomidine, etomidate, metoclopramide, atracurium, and others are examined in detail.

Natural Pesticides: Methods. Edited by B. Mandava. Published by CRC Press, Inc., 2000 Corporate Blvd, NW, Boca Raton, FL 33431, Spring 1985. 592 pp. Price: $108.00 (prepub., U.S.)/ $125.00 (outside U.S.). ISBN 0-8493-3651-1.

Volume I: Theory, Practice, and Detection explains pest management practices, discusses allelopathy and allelochemicals, and examines how insects depend on sensory chemicals for reproduction. Also presented are methods for detection. Regulations and registration requirements for pesticides are reviewed.

Polynuclear Aromatic Compounds. International Agency for Research on Cancer (IARC), Lyon, France. Distributed by the World Health Organization, 1211 Geneva 27, Switzerland, 1984. 245 pp. Price: $25.00 (U.S.)/SFr. 60.-. ISBN 92-832-1533-9 (soft-bound); ISBN 92-832-1533-8 (hard-bound).

Volume 33 of the IARC Monographs of the Carcinogenic Risk of Chemicals to Humans contains evaluations of carbon blacks, mineral oils, and some nitroarenes. Also included are evaluations of carcinogenicity of both carbon black particles and solvent extracts of carbon blacks.

CRC Handbook of HPLC for the Separation of Amino Acids, Peptides, and Proteins. Edited by W. S. Hancock. Published by CRC Press, Inc., 2000 Corporate Blvd, NW, Boca Raton, FL 33431, 1984. 512 pp. Price: $92.00 (prepub., U.S.)/$106.00 (outside U.S.). ISBN 0-8493-3511-6.

Volume I introduces the general principles of HPLC and covers HPLC instrumentation, mobile phases, detection methods, separation of free amino acids, resolution of amino acids as diasteriomeric derivatives, and application of ligand-exchange chromatography to separation of amino acids, peptides, and proteins.

How To Comply With The New Copyright Law

FOR YOUR INFORMATION

A New Year and a New President for AOAC—Richard J. Ronk of FDA

Richard J. Ronk, deputy director of the Food and Drug Administration (FDA) Center for Food Safety and Applied Nutrition, will serve as AOAC (Association of Official Analytical Chemists) president for 1985.

As deputy director since 1980, Mr Ronk provides overall administrative and management direction for the center through his involvement in planning financial and general resource management, program analysis evaluation, and development and operation of appropriate scientific and program management support systems.

Mr Ronk has been director of the Division of Food and Color Additives, Office of Compliance; chief of the Guidelines and Compliance Research Branch, Regulatory Guidance Division; Food and Drug Officer for the Regulatory Guidance Division, Bureau of Compliance; and an FDA chemist. He has also served as U.S. Delegate to the Food Additives Committee of the Codex Alimentarius Commission.

Richard Ronk received both BA and MS degrees from Creighton University. He is vice president of the Toxicology Forum and is a member of the American Chemical Society. He has received the FDA Award of Merit for the mercury in fish program in 1971 and the Superior Service Award for Public Health Service in 1977.

Summaries of Collaborative Studies Conducted by Other Organizations

AOAC is initiating a new feature of summarizing the results of collaborative studies conducted by other organizations. Although AOAC may have been the first organization to insist on the use of interlaboratory trials to validate the performance of methods of analysis, many other organizations now are seeing the necessity for determining the intra- and interlaboratory performance characteristics of methods. There is no need

to publish methods of analysis if they are not intended for use by other laboratories. As soon as 2 laboratories are involved in the analysis of the same lot of goods, whether it be for scientific, economic, or legal reasons, there is a need to know how well they are expected to agree with each other. Collaborative studies provide the estimate of expected agreement, and with a suitable experimental design, an estimate of how well the results should agree with the correct, assumed, or required composition.

To initiate this series, the International Office of Vine and Wine provided an extensive study of the fundamental determinations used in the examination of wines: mass per unit volume (density), alcohol content, reducing sugars, and total and volatile acidity. The summary is published in this issue. The organization represents most of the viticultural countries in the world. A subcommission was established about 50 years ago to unify the methods of analysis and characteristics of wines. They publish a book of those methods (200 French francs) and a journal (300 French francs/year). The address of the organization is: Office International de la Vigne et du Vine, 11, rue Roquepine, Paris (8ᵉ), France.

The results of collaborative studies from other organizations in any commodity area may be submitted for consideration for inclusion in this section. These may or may not have been previously published, but in all cases, a copy of the original data (individual determinations, not averages), carefully checked to ensure freedom from typographical errors, must be submitted Although all studies will be presented as interpreted by the administrator of the study, the original data will be further analyzed by the program FDACHEMIST, or a subsequent version, in order to place the results from all studies on a consistent statistical basis An explanation of the program (written in APL language) and an example of its use will be found in the article "Performance of Methods of Analysis Used for Regulatory Purposes. I. Drug Dosage Forms" (*J. Assoc. Off. Anal. Chem* (1984) **67**, 81–90). Comparing the interpretations of the administrator of the study with that obtained by the statistical program may provide a basis for harmonizing the interpretation of results from collaborative studies.

Candidate studies for inclusion in this series should be sent to William Hor-

witz, Center for Food Safety and Applied Nutrition, HFF-7, Food and Drug Administration, Washington, DC 20204 USA. Such studies should meet the current recommendation of the Committee on Collaborative Studies for the design of a minimum collaborative study: (number of materials × number of laboratories) = 30, with a minimum of 5 laboratories (and an even number of materials, if Youden pairs are used), with single determinations by each laboratory. However, the collection of considerably more data is encouraged.

Submission of complete papers describing collaborative studies performed by other organizations for consideration for publication in full in the *Journal of the Association of Official Analytical Chemists* is an alternative which is also available.

EPA Project Summary

Eighteen laboratories participated in a study of EPA Method 609 for measuring concentrations of the Category 4 chemicals nitrobenzene, isophorone, 2,4-dinitrotoluene, and 2,6-dinitrotoluene in municipal and industrial aqueous discharges. Method 609 involves solvent extraction of the pollutants with methylene chloride, followed by Florisil cleanup and subsequent gas chromatographic analysis of the 4 subject compounds, using flame ionization and electron capture detection techniques.

The study design was based on Youden's plan for collaborative tests of analytical methods. Three Youden pair samples of the test compounds were spiked into 6 types of test waters and then analyzed. In general, mean recoveries, overall standard deviations (S), and the single-analyst standard deviations (SR) were directly proportional to the true concentration levels. There were no discernible differences due to water types among mean recoveries, overall precisions, and single-analyst precisions.

Based on the results of the interlaboratory method study, Method 609 is a viable analytical method for measuring concentrations of the Category 4 chemicals in industrial wastewaters. Use of Method 609 by experienced analysts should enable industries to meet the requirements of the NPDES program for discharging the subject pollutants into the environment.

The complete report (order No. PB 84-176 908) can be obtained for $11.50 (subject to change) from the National

Technical Information Service, 5285 Port Royal Rd. Springfield, VA 22161; 703/487-4650. For more information, contact: Edward L. Berg and Robert L. Graves, Environmental Monitoring and Support Laboratory, U.S. Environmental Protection Agency, Cincinnati, OH 45268.

Texas Instruments Foundation Memorial Prize

Nominations for the Texas Instruments Foundation Founders' Prize of $50,000 (tax free) to be awarded in the spring of 1985 are now being accepted.

This memorial prize is awarded to recognize outstanding individual achievement in the physical sciences, health sciences, management sciences, engineering, or mathematics. For more information concerning the prize and nominations, contact: Mr. S. T. Harris, M.S. No. 232, chairman, Founders' Selection Committee, Texas Instruments Foundation, PO Box 22574, Dallas, TX 75265.

Standard Reference Materials

The National Bureau of Standards (NBS) Office of Standard Reference Materials announces the availability of the following Standard Reference Materials (SRM): SRM 640a, a high purity silicon powder, features a mean particle size of about 2 μm, lattice spacing of 5.430825 Å, twelve 2θ values (CuKα radiation) ranging from 28.443 to 158.644°, and relative intensities for constant volume are given for 11 diffraction lines. Price: $83/10g unit.

SRM 675, a synthetic fluorophlogopite mica, ground to pass a 75 μm sieve, features lattice spacing of 9.78104 Å, 2θ values (CuK α radiation) ranging from 8.853 to 135.574°, and relative intensities given for 11 diffraction lines. Price: $105/5 g unit.

SRM 674, a set of 5 powders (Al_2O_3, ZnO, TiO_2(rutile), Cr_2O_3, and CeO_2). Price: $132 per set (20 g of each powder).

SRM 1878, Respirable Alpha Quartz, features size distribution in the inhalable/respirable range (0.3–5 μm). Price: $147/5 g unit.

SRM 493, Spheroidized Iron Carbide (Fe_3C) in Ferrite, is used for the calibration of X-ray diffraction and Mossbauer equipment. Price: $133/wafer.

The following 4 Standard Reference Materials (in the form of disks) are used for assessing X-ray diffraction techniques used in determining the amounts of retained austenite in ferrous materials: SRM 485a, 5% Austenite in Ferrite, $290; SRM 486, 15% Austenite in Ferrite, $301; SRM 487, 30% Austenite in Ferrite, $290; and SRM 488, 2.5% Austenite in Ferrite, $283.

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

Short Courses

The State University of New York at Albany will offer 2 short courses in the summer 1985: X-Ray Powder Diffraction, June 17–28; and X-Ray Spectrometry, June 3–14. Both courses deal with the basic theories and techniques on the subject matter. For more information, contact: Professor Henry Chessin, State University of New York at Albany, Dept of Physics, 1400 Washington Ave, Albany, NY 12222; 518/457-8339.

The American Oil Chemists' Society will offer 2 short courses: Processing and Quality Control of Fats and Oils, April 30 and May 1, 1985; and Applications of Analytical Methodology in Fats and Oils Processing, May 2–4, 1985. For more information, contact: Meeting Coordinator, American Oil Chemists' Society, 508 S 6th St, Champaign, IL 61820.

Meetings

April 8–11, 1985: 10th Annual AOAC Spring Training Workshop, Sheraton Hotel, Dallas, TX. Contacts: M. Virginia Gibson, FDA, 3032 Bryan, Dallas, TX 75204, USA, 214/767-0312; or Molly Ready, Alcon Labs, 6201 S Freeway, Fort Worth, TX 76134, USA, 817/293-0450.

April 11–12, 1985: American Association of Cereal Chemists' Oak Ridge Conference on Advanced Analytical Concepts for Clinical Laboratory, Sheraton Charleston, Charleston, SC. Contact: Meetings Dept, AACC, 1725 K St, NW, Washington, DC 20006, USA, 202/857-0771.

May 2–4, 1985: American Oil Chemists' Society Research Conference on Fat Requirements for Development and Health, Hershey Poconos, White Haven, PA. Contact: Meetings Coordinator, American Oil Chemists' Society, 508 S 6th St, Champaign, IL 61820, USA, 217/359-2344.

May 5–9, 1985: 76th Annual Meeting of the American Oil Chemists' Society, Wynham Franklin Plaza Hotel, Philadelphia, PA. Contact: Meetings Coordinator, American Oil Chemists' Society, 508 S 6th St, Champaign, IL 61820, USA, 217/359-2344.

June 17–19, 1985: AOAC Midwest Regional Section Meeting, Joliet, IL. Contact: Devendra Trivedi, Illinois Law Enforcement Dept, Bureau of Scientific Services, 515 E Woodruff Rd, Joliet, IL 60432, USA, 815/727-5301.

June 23–26, 1985: The Canadian Institute of Food Science and Technology's 28th Annual Conference, Royal York Hotel, Toronto, Ontario. This year's theme, "Food Links for Progress," is designed to provide a platform for dialogue to food professionals around the world so that we may obtain a better understanding of how we can mutually benefit from working together. Other features include: technical sessions, social events, companion programs, student activities, and exhibit booths. Contacts: Tena Koornneef, Marketing and Publicity, Foodpro National Inc., 1589 The Queensway, No. 2, Toronto, Ontario, Canada M8Z 5W9, 416/252-7286; or Bill Munns, Conference chairman, Canada Packers Inc., 95 St. Clair Ave W, Toronto, Ontario, Canada, M8Z 1P2, 416/766-4311.

June 24–27, 1985: Medical Device and Diagnostic Industry's 4th Conference Expo., O'Hare Convention Center, Chicago, IL. Topics will include: computer hardware/software validation systems; current regulatory affairs; drug-delivery systems; device failure analysis; as well as state-of-the-art information on other manufacturing, business, and marketing topics. Contact: Expocon Management Associates, 3695 Post Rd, South Port, CT 06490, USA, 203/259-5734.

July 21–26, 1985: 37th Annual Meeting of the American Association for Clinical Chemistry, Georgia World Congress Center, Atlanta, GA. Contact: Meetings Dept, AACC, 1725 K St, NW, Washington, DC 20006, USA, 202/857-0717.

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

November 3–8, 1985: World Conference on Emerging Technologies in the Fats and Oils Industry, Palais des Festivals, Cannes, France. Contact: Meetings Coordinator, American Oil Chem-

ists' Society, 508 S 6th St, Champaign, IL, USA, 217/359-2344.

November 4–10, 1985: AG China 85 (the first China agricultural exhibition), Beijing National Agricultural Exhibition Center, Beijing, China. Topics will include: agricultural technology and related products, processing equipment in the field, fertilizers and other agricultural chemicals, equipment for the small farmer, and products for state farms. Contact: CEG/International, Cahners Exposition Group, PO Box 70007, Washington, DC 20088, USA, 301/657-3090.

April 13–16, 1986: 11th Annual AOAC Spring Training Workshop, Madison Hotel, Seattle, WA. Contact: Mike Wehr, Oregon Dept of Agriculture, Agriculture Bldg, 635 Capital St, NE, Salem, OR 97310, USA, 503/378-3973.

July 20–26, 1986: SAC 86 International Conference on Analytical Chemistry and the Biennial National Atomic Spectroscopy Symposium, University of Bristol, Bristol, England, is organized by the Analytical Division of the Royal Society of Chemistry in conjunction with the Spectroscopy Group of the Institute of Physics. Contact: The Secretary, Analytical Division, Royal Society of Chemistry, Burlington House, London W1V OBN, UK.

August 10–17, 1986: 6th International Congress of Pesticide Chemistry, Ottawa, Ontario, Canada, is sponsored by the International Union of Pure and Applied Chemistry. Main topics deal with the chemical, biochemical, physiochemical, analytical, and regulatory aspects of pesticide science. Contact Chemical Institute of Canada, 151 Slater St, Suite 906, Ottawa, Ontario, Canada K1P 5H3.

New Private Sustaining Members

AOAC welcomes 3 new private sustaining members to the growing list of firms aware of the need to support an independent methods validation association: Jack Daniel Distillery, LEM, Motlow, Prop., Lynchburg, TN; Conagra Consumer Frozen Foods Co., Columbia, MO; Chemical Waste Management, Inc., Riverdale, CA.

Reminder of Deadlines for Nominations for Awards

The following deadlines have been set for nominations for awards: 1985 Fellow of the AOAC Award, March 1, 1985; and 1985 Harvey W. Wiley Award, April 1, 1985.

The Fellow of the AOAC award was established in 1961 to recognize those persons giving meritorious service to the Association. Eligible candidates have performed notably for 10 years or more, usually as Officers, Referees, or Committee members. Fellows are selected by the Committee on Fellows and approved by the Board of Directors. Selection is made from a list which includes eligible candidates, their contributions, and the point count assigned to each candidate according to an established point system. The Committee is also informed of any nominations received. Contact: Margaret R. Ridgell, Administrative Manager, AOAC, 1111 N 19th St, Suite 210. Arlington, VA 22209, USA; 703/522-3032.

Corrections

J. Assoc. Off. Anal. Chem. (1984) **67**, 1024–1026,

"Quantitative Amino Acid Analysis of Feedstuff Hydrolysates by Reverse Phase Liquid Chromatography and Conventional Ion-Exchange Chromatography," p. 1024 right column,

Change the sentence which begins with "The present study . . ." to read, "The present study was conducted to quantitatively compare a recently developed reverse phase LC technique with a conventional IEC procedure for the amino acid analysis of corn, soybean, and isolated soybean protein, 3 common feedstuffs of low, intermediate, and high protein content, respectively."

INFORMATION FOR SUBSCRIBERS, CONTRIBUTORS, AND ADVERTISERS

REVIEW OF ASCORBIC ACID METHODOLOGY

Analytical Methods for Determining Ascorbic Acid in Biological Samples, Food Products, and Pharmaceuticals

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Over the last decade, numerous publications have appeared describing analyses for ascorbic acid in food products, pharmaceuticals, and biological samples. This review focuses on the chemistry associated with many of these procedures. The papers discussed have historical importance, are important to understanding the method, or have significantly advanced ascorbic acid analysis. The review has 4 major sections: spectroscopic, electrochemical, enzymatic, and chromatographic methods of analysis.

Ascorbic acid is an important vitamin having a chemical structure that justifies its classification as a carbohydrate. Its physical/chemical properties, compiled by Kutsky (1), are as follows: aqueous solubility = 0.3 g/mL, melting point = 190–192°C, redox potential = E_o = 0.166 V at pH 4, pKa = 4.17, pKa_2 = 11.57, and absorption maxima = 245 nm (acid) and 265 nm (neutral). Ascorbic acid is rapidly oxidized to dehydroascorbic acid (DHAA) by oxygen and metal ions in alkaline and acidic media. Dehydroascorbic acid can be further oxidized to diketogulonic acid. The structures of these 3 compounds are shown below:

analysis (3, 4). A symposium on ascorbic acid, its chemistry, metabolism, and uses, was sponsored by the Division of Carbohydrate Chemistry at the Second Chemical Congress in 1980. At this symposium, Sauberlich and colleagues presented a review that discussed the advantages of liquid chromatographic (LC) methods over spectroscopic methods (5). *Methods in Enzymology* has also devoted a section in a recent volume to ascorbic acid (6).

Recently, Cooke and Moxon have reviewed spectroscopic and chromatographic methods of analysis for this vitamin in food products (7). That review does not include electrochemical or enzymatic methods of analysis.

This review surveys the analysis for ascorbic acid in pharmaceuticals, food products, and biological samples, and focuses on the chemistry associated with many of these procedures. A review of stability or sample extraction has not been included, because this topic is discussed in cited papers or the above reviews. Only selected papers of historical importance have been cited, those which are important to the chemical under-

Ascorbic Acid   Dehydroascorbic Acid   Diketogulonic Acid

Over the last decade, numerous publications have appeared describing analyses for ascorbic acid in food products, pharmaceuticals, and biological samples. This resurgence in new analytical procedures results from the importance of the vitamin in nutritional, clinical, pharmacological, and industrial studies. A monograph containing selected procedures for pharmaceutical preparations has appeared (2). In addition, a review on the determination of this vitamin in pharmaceuticals has been included in biannual updates on pharmaceutical

standing of the method, or important in advancing the selective, sensitive analysis for ascorbic acid. The review has been divided into 4 major sections: spectroscopic, electrochemical, enzymatic, and chromatographic methods of analysis.

Spectroscopic Methods

The classical chemistry associated with spectroscopic methods can be divided into 2 general categories: (a) those using a redox indicator in its oxidized form, and (b) those

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involving chromogen formation by derivatization. The redox indicators that will be discussed include 2,6-dichloroindophenol, metal ions, and other miscellaneous reagents. The derivatization of ascorbic acid includes reaction with 2,4-dinitrophenylhydrazine, diazotization, and quinoxaline formation.

Redox Reactions

2,6-Dichloroindophenol.—The standard redox reagent used for ascorbic acid analysis in a variety of sample types is 2,6-dichloroindophenol (DCIP). Solutions of the reagent are blue at neutral pH and pink in acid. The stoichiometry of the redox reaction was first proposed by Tillmans (8). The reaction is rapid and is first order with respect to each reactant. The overall second order rate constant was pH-dependent with an optimum value of 56.5×10^3 L/mole/s at low pH (9, 10).

phenol, and therefore interfere. Attempts to minimize these positive biases have included chromatographic isolation of ascorbic acid (22) and heavy metal ion removal via mercaptide salt precipitation before the reaction (23). Another way to improve the specificity involves following the kinetics of the reaction between the vitamin and DCIP. Hiromi and coworkers used the linear relationship between the apparent first order rate constant for reduction of the indophenol to determine ascorbic acid concentration (24, 25). The reaction of excess ascorbic acid with DCIP was linear between 0.5 μM and 50 μM and was suitable for analysis of orange juice. Cysteine and glutathione did not interfere, and interference by triose reductone was minimized. Recently, these investigators improved the lower detection limit to 2 μM by reacting ascorbic acid in the presence of excess reagent and using a stop-flow kinetic procedure (26). This method was applied to

The first application of 2,6-dichloroindophenol to ascorbic acid analysis was reported in 1932 by Tillmans et al. (11). After numerous modifications, the method still consists of monitoring DCIP absorbance at 518 nm before and after sample addition. Hoffman et al. have automated the DCIP procedure and used it to determine the concentration of ascorbic acid in orange and grapefruit juices (12). Their method was unsuitable for the assay of lemons because of the high acidity of the sample. Sample turbidity was minimized by dialyzing the ascorbic acid from the sample directly into the flowing DCIP solution. Another automated version introduced by Egberg and coworkers was applied to the assay of fortified grains and beverages (13). This method required a methanolic–metaphosphoric acid extraction step to reduce turbidity from colloidal suspensions. The detection limit of the method was 6 μg/mL and response was linear up to 200 μg/mL.

There has been considerable interest in determining ascorbate concentrations for nutritional and biomedical surveys. Because of the large number of samples in these studies, several variations of the basic methodology have been developed. Methods have been introduced for cataractous lens (14), ovarian (15), and lingual tissues (16); infant milk (17); and serum, whole blood, and saliva (18–20). One automated version, rigorously evaluated by independent investigators, has been chosen as a "selected method" for clinical laboratories (21). This method produces a linear response from 2.0 to 20 μg ascorbic acid/mL serum, with a maximal sampling rate of 60/h. Interferences from sulfhydryls, sulfites, and thiosulfates and from catalytic oxidation of ascorbate by iron(III) and copper(II) were minimized by using a pH 3.5 solution of metaphosphoric acid.

The limitations encountered with DCIP methods result from poor specificity and dye instability. Many molecules (e. g., phenols, sulfhydryls, and triose reductones) and ions (e.g., ferrous, cuprous, or sulfite) can reduce 2,6-dichloroindo-

a variety of vegetables and fruits and requires only 1–2 s/sample. The suitability of this type of methodology depends on advances in stop-flow methods (27) and a need to analyze many samples. This methodology should find acceptance in clinical laboratories with centrifugal analyzers.

Metal Ions.—Another class of colorimetric redox reactions involves the reduction of metal ions to produce a stable colored solution. For example, ascorbic acid may be determined in pharmaceutical preparations by using potassium ferricyanide (28) or ferricinium trichloroacetate (29). Pelizzeti. et al. (30, 31) have reported the kinetics and mechanisms between ascorbic acid and many potentially useful metal-ion complex oxidants.

The more common metal-ion redox methods involve the reduction of iron(III) to iron(II) by the vitamin. An intensely colored iron(II) complex is formed after addition of a chelating agent. Absorbance is monitored at the absorption maximum of the complex and is directly proportional to the ascorbic acid concentration. The most common iron(II) chelating agents are α,α'-dipyridine, 2,4,6-tripyridyl-*S*-triazine (TPZ), and ferrozine.

Zannoni and coworkers have published a micromethod for the determination of ascorbic acid in plasma and tissue samples (32). This procedure detects as little as 0.1 μg ascorbic acid and requires 0.01 mL sample; the complex is stable for at least 2 h. Okamura has reported a procedure for the simultaneous determination of ascorbic and dehydroascorbic acids in plasma (33, 34). In both procedures, dehydroascorbic acid is reduced to ascorbic acid with dithiothreitol. The ascorbate and dehydroascorbate concentrations are then determined by difference. Precipitation of iron from urine samples and treatment with activated charcoal to remove interfering substances were necessary. Excellent agreement was found between this procedure and a dinitrophenylhydrazine method.

Lloyd et al. have used 2,4,6-tripyridyl-*S*-triazine to develop a procedure for analysis for ascorbic acid in platelets (35).
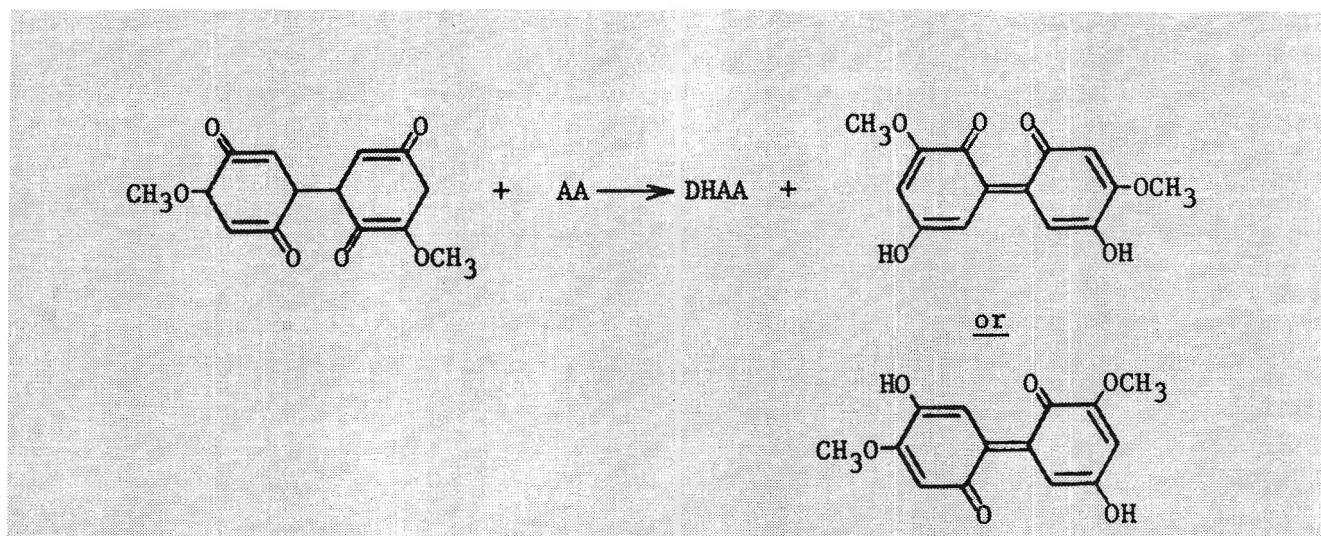
The platelets are extracted and the supernate is chromatographed on cellulose TLC plates. Ascorbic acid was eluted and reacted with iron(III) in the presence of the complexometric reagent. The precision of this method was 2.5%. This reagent was also suitable for the analysis of plasma samples (36). Acidification of plasma followed by reaction with iron(III) and treatment with chelating reagent are the only necessary treatment steps. This procedure was applicable to clinical samples.

Another excellent iron(II) complexing agent in widespread use is ferrozine (3-(2-pyridyl-5,6-bis(phenylsulfonic acid)-1,2,4-triazine)). The advantages of ferrozine over other Fe(II) reagents include water solubility, stability, and a higher molar absorptivity (27 900) when compared with phenanthroline (11 000) or α,α'-bipyridyl (8650) complexes. The kinetics of the reaction between iron(II) and ferrozine, 1-10-phenanthroline, and α,α'-bipyridine have been compared (37). Jaselskis and Nelapaty have characterized the optimal reaction conditions and have developed a method suitable for the quantitation of ascorbic acid in citrus fruits (38). A pH 3–6 solution was optimal for color formation, and as little as 0.2 μg ascorbic acid/mL could be quantitated. Butts and Mulvihill have used this chromogenic reagent to develop a procedure for determining the vitamin in serum or urine (39). Their procedure was based on rapid formation of the ferrozine complex, using the unique ability of a centrifugal analyzer to simultaneously measure standards and samples (and a blank if necessary) after very short reaction times (>15 s). By carefully optimizing the pH, reagent composition, and reaction time, they were able to successfully assay for ascorbic acid in the presence of uric acid, sulfhydryls, and other reductants. Recently, this procedure has been modified for manual determination of the vitamin in tissue samples (40).

Other reported metal-chelometric procedures include the formation of an iron(II)–bathophenanthroline complex to determine ascorbic and dehydroascorbic acids in rat tissue samples (41), and formation of a copper(I)–2,2 bisquinoline complex which is the basis of a method for pharmaceutical preparations (42). In general, the redox chelometric methods have the same disadvantages as DCIP methods. Because the vitamin is measured indirectly, the presence of other reducing agents will positively bias the results.

Miscellaneous Reagents.—Several other redox reagents have been used for determining ascorbic acid in biological fluids, pharmaceuticals, and food products. One method for pharmaceutical preparations is based on the production of an intense red-violet color when dimethoxyquinone is reduced

by ascorbic acid (43). The molar absorptivity of the product is 1738 at 510 nm and Beer's law is followed for ascorbate concentrations between 10 and 80 μg/mL. This reagent has been modified and applied to the quantitation of the vitamin in citrus fruits (44). The reaction has been characterized (45) and is complete within 6 min at 25°C for amounts of ascorbic acid as high as 0.8 mg. The reaction product has been synthesized and characterized by infrared, nuclear magnetic resonance, and mass spectrometric techniques. The reduced dimethoxyquinone was present as 2 isomeric products.

Kinetic analytical methods have appeared that use bromine generated from the reaction between bromate and bromide in acidic solution (46, 47) or oxidation of ascorbic acid by iodate (48). White and Fitzgerald used methylene blue to analyze ascorbic acid tablets and fruit juices (49). The reduction of perinaphthindan-2,3,4-trione at pH 3 with subsequent monitoring of the reduced chromogen at 460 nm was the basis of another method used for citrus fruits (50). The ease of determining the titer of standard ascorbate solution has been facilitated by using o-toluidine (51), and phosphotungstic acid has been used as the basis of a serum ascorbate method (52). Two phosphotungstic acid methods have been published for the determination of ascorbic acid in pharmaceuticals, plasma (53), and vegetables (54). Folin phenol reagent has been used for determining ascorbic acid in biological samples (55).

Derivatization Reactions

Dinitrophenyl Hydrazine.—Another classical colorimetric method involves measuring the absorbance produced when dinitrophenylhydrazine (DNPH) couples with the oxidized form of ascorbic acid. In this procedure (commonly termed the Roe and Kuether method) (56), ascorbic acid is first converted to dehydroascorbic acid (DHAA) via a suitable oxidizing agent. Dinitrophenylhydrazine is added and, after a brief incubation time, color is produced after acidification with concentrated sulfuric acid. The absorbance is measured at 520 nm and is proportional to the original ascorbic acid and DHAA concentrations.

The specificity of the method is attributed to the following factors: *(1)* color is produced more easily with 2,4-dinitrophenylhydrazine derivatives of 5- and 6-carbon, sugar-like compounds; *(2)* the rate of coupling is much faster with dehydroascorbic acid than with other carbohydrates; and *(3)* measurable chromogen formation from non-ascorbic acid substances is minimized by carrying out the reaction at low temperatures. Zloch and coworkers (57) have evaluated the Roe and Kuether method using thin layer chromatography
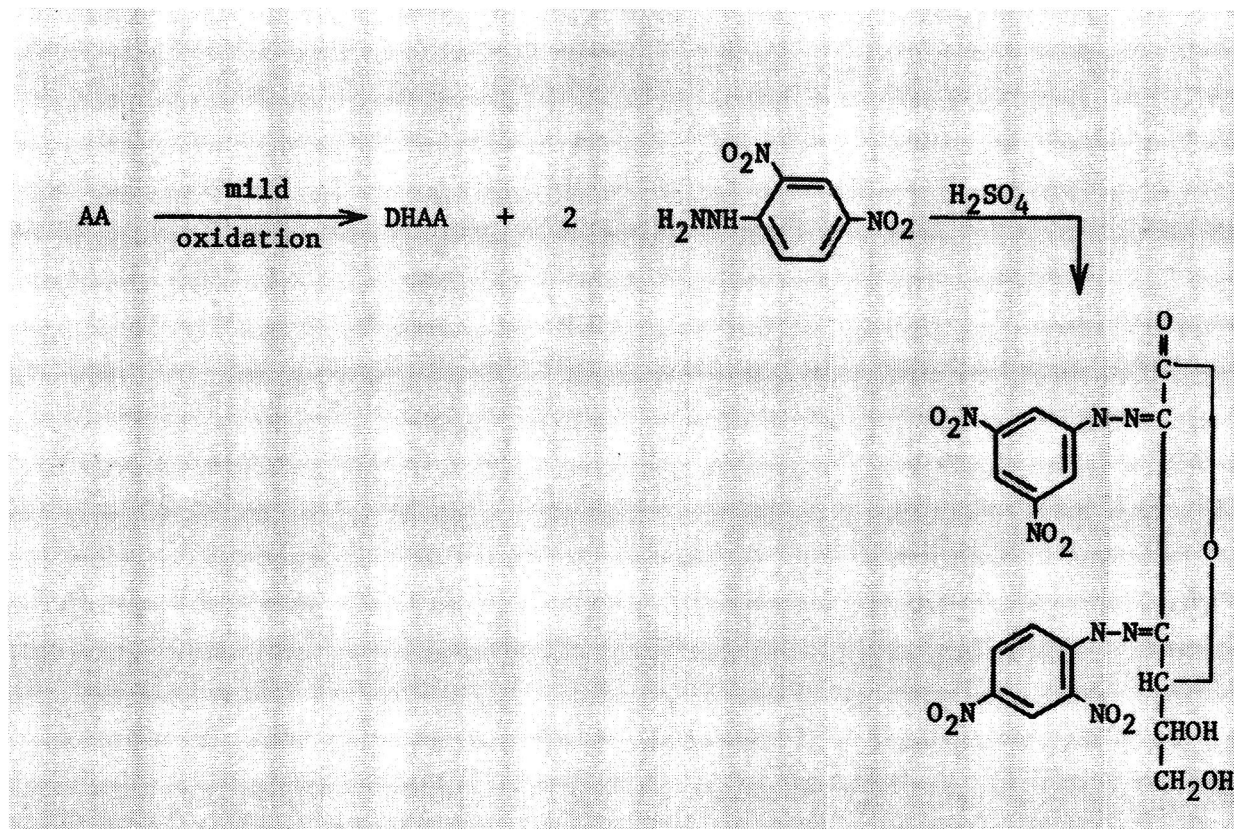
and ^{14}C-labeled ascorbic acid and found that only 30–53% of the bis-2,4,-dinitrophenylhydrazone derivative was produced. Optimal derivatization conditions yielding maximal derivative formation were 3–4 h at 37°C, or 1.5 h at 60°C. This approach has been used to monitor urinary ascorbate levels (58).

A method has been reported, using the oxidant potassium bromate, that can quantitate ascorbic acid and its important metabolite, ascorbic acid-2-sulfate, in urine and tissues (59). Free ascorbic acid is quantitated after 1 h incubation at 37°C, although its metabolite is measured after incubation for 3 h at 60°C. The difference in absorbance at both temperatures corresponds to the initial amount of ascorbic acid-2-sulfate.

Many investigators have integrated selective chromatographic techniques to increase specificity. For example, Beljaars et al. combined thin layer chromatography with the 2,4-DNPH method (60). They applied densitometric detection for measuring 0.08–1.00 µg ascorbic acid in buttermilk. Shmidt and Holfelder used thin layer chromatography to isolate the 2,4-dinitrophenylhydrazone derivative from interfering components and were able to quantitate the ascorbic acid content in black current juice (61). Micro and macro methods using this approach were developed to determine ascorbic acid levels in body fluids and tissues of insects (62).

Toothill and coworkers incorporated column chromatography and compared their results with a 2,6-dichloroindophenol method for ascorbic acid analysis in evaporated and fortified sterilized milk (63); they reported the DPNH method to be more accurate. The presence of sulfhydryl groups and reductones present in evaporated and sterilized milk interfered in the 2,6-dichloroindophenol method, but not in the TLC/DPNH method. Dried feeds have been assayed for ascorbate content with combined column chromatography and the DPNH method (64). This approach has a throughput of 50–60 samples/day and a detection limit of 0.1 µg ascorbic acid/g. Sample cleanup on a Dowex 1-X2 anion-exchange

column has also been used for quantitation of ascorbic, dehydroascorbic, and 2-ketogulonic acids (65). A 50 mM H_3PO_4 mobile phase was optimal.

The DPNH method has been automated by Aeschbacher and Brown and applied to analysis of tissue samples (66). A manual method has also been reported for analysis of platelets and leukocytes for ascorbic acid (67). Serious disadvantages of the manual methods include lengthy derivatization steps and the fact that DHAA is also measured as ascorbic acid. Pelletier has reported a combined manual method incorporating dichloroindophenol and dinitrophenylhydrazine to differentially measure ascorbic acid and DHAA levels in biological samples (68). An automated version of this methodology has been adapted for determining ascorbic acid in serum (69) and pharmaceutical products (70). An in-depth investigation of the automated experimental parameters has been undertaken and applied to foodstuffs (71). The parameters investigated included maximization of the conversion of DHAA to ascorbic acid for blanks, minimization of osazone formation by sugars, and optimization of color development. The procedure was applied to vegetables, fruit juices, and other food products. The method was more specific when compared with a manual DPNH method. Behrens and Madere (72) have made further improvements in Pelletier and Brassard's method (69). Refinements in the reaction conditions and choice of reagents allowed determination of ascorbic acid in small volumes of rat plasma and tissue extracts. The new procedure requires only 0.15 mL sample and could measure as little as 1.2 µg/mL. Use of the method yielded standard deviations of 0.5–1.5% for ascorbate concentrations between 1.6 and 6.8 µg/mL.

Wahba and coworkers developed a simplified colorimetric assay to determine ascorbic acid in pharmaceutical preparations (73). This method consists of reacting ascorbic acid with phenylhydrazinium chloride in acidic solution at 50°C, with subsequent measurement at 395 nm. The assay obeys Beer's

law from 25 to 100 μg, and interference by other vitamins and reducing substances is minimized.

Diazotization.—Another class of derivatization methods involves the reaction of ascorbic acid with diazotized 4-methoxy-2-nitroaniline. First studied by Schmall and coworkers (74, 75), the reaction mechanism is a hybrid redox-derivatization and is illustrated below.
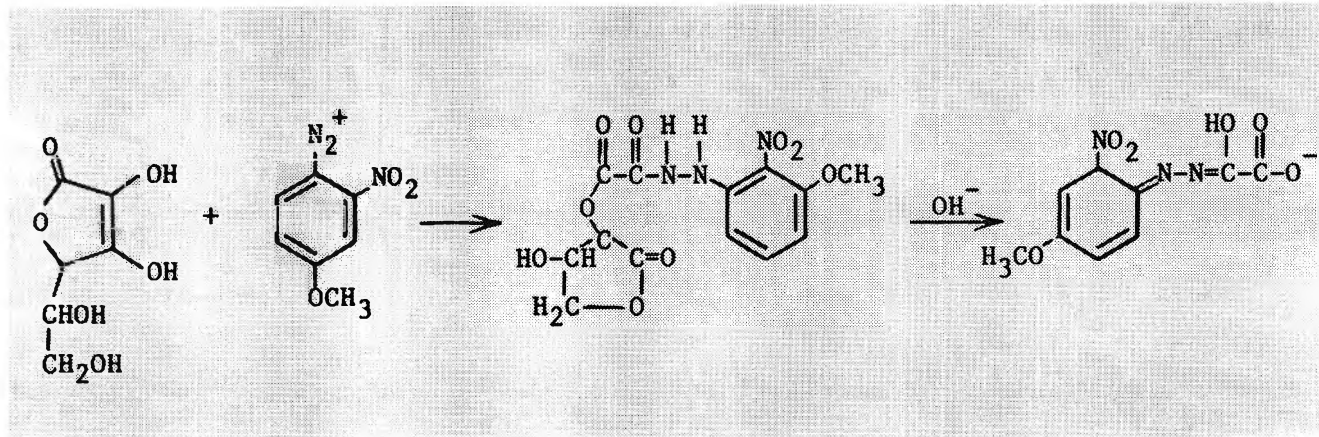
Derivatization is rapid, and the absorbance of the alkaline blue solution (570 nm) is proportional to the original ascorbic acid concentration. Other vitamins and dehydroascorbic acid do not react with the diazotization reagent. This methodology is, therefore, appropriate for pharmaceutical stability studies.

The basic manual procedures have been modified and adapted to a Technicon Autoanalyzer for automated serum and urinary ascorbate analysis (76). The automated method obeys Beer's law over the concentration range of 1–7 mg/100 mL, and specificity is enhanced for biological samples by using blank correction. Diazotized 4-methoxy-2-nitroaniline has also been used in an ovarian ascorbic depletion assay (77) and more recently in an automated pharmaceutical formulation assay (78). Chromatography on a cellulose column has been used for sample cleanup for selective determination of ascorbic acid in foodstuffs (79). Other variations of the method use *p*-nitroaniline (80) and/or diazotized *p*-aminobenzoic acid as the redox-derivatization reagent (81).

Quinoxaline Formation.—Alternative derivatization reactions include the formation of condensation products derived between dehydroascorbic acid and substituted *o*-phenylenediamines (OPDA):
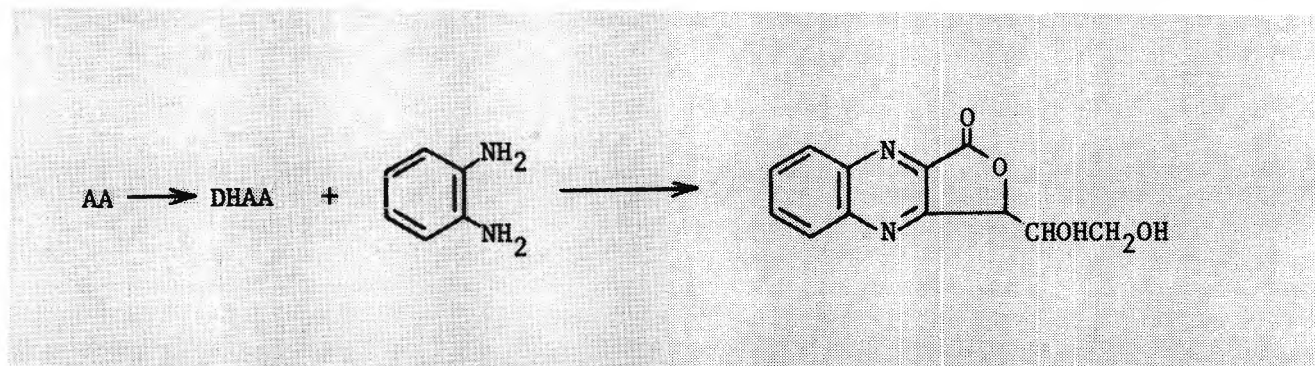
The manual OPDA method has been automated on Technicon Autoanalyzer by simply replacing the Norit oxidation step with redox reagents (84–86), but a major drawback of using redox reagents in a totally automated system is that naturally occurring fluorescing plant components may interfere because they are no longer adsorbed onto the Norit. This problem can be minimized by using a semiautomated version (87).

The automated method of Roy et al. (85) and semiautomated method of Egberg et al. (87) have been compared (88) with 2 manual AOAC titrimetric and fluorometric methods (89, 90). In this study, 40 products (cereals, fruits, vegetables, baby foods, meats, frozen dinners, juices, nutritional health bars, and pet foods) were assayed in duplicate on 2 days and included a recovery study. Egberg's method was found to have a correlation factor of 0.999 with the official methods and an average recovery of 97.8%; while the Roy method had a correlation factor of 0.979 and an average recovery of 99.3%. On the basis of these results, it has been suggested that Egberg's method is the method of choice for most of the products tested. A recent study has determined the within- and between-laboratory reproducibilities of Egberg's semiautomated method (91). Fifteen samples of 12 different products (cereals, fruit juices, and infant formula) were assayed by 5 different laboratories. The within-laboratory relative standard deviations for 3 different blind duplicate samples were 11.9, 0.93, and 3.2%. The average relative standard deviation between laboratories was 4.9% (range 1.5–12.6%). On the basis of these results and the historical performance of the method (88, 91), the semiautomated version was adopted as

The most commonly used method is the manual Deutsch and Weeks assay (82). Norit (carbon) oxidizes ascorbic acid to DHAA which then reacts with *o*-phenylenediamine to yield a fluorescent quinoxaline derivative. This method is more specific, faster, and less restrictive to sample type when compared with the dichloroindophenol and DPNH assays (83).

an official method at the 97th annual international meeting of AOAC.

Other variations of the basic *o*-phenylenediamine approach have used substituted analogs of OPDA in the reaction. For example, Szepesi used 4,5-dimethyl-1,2-phenylenediamine (92). This new derivatization reagent allowed the ascorbic

acid content of pharmaceutical preparations to be determined by ultraviolet spectroscopy, and a 4-fold decrease in derivatization time was reported.

Another spectrophotometric method that incorporates an analog of OPDA, 4-nitro-*o*-phenylenediamine, has been used for analyzing ascorbic acid in foodstuffs (93) and pharmaceutical preparations (94). These approaches consisted of retaining ascorbic acid on an anionic Sephadex column. After interfering substances were eluted, ascorbic acid was oxidized to dehydroascorbic acid in situ with *p*-benzoquinone. Dehydroascorbic acid was eluted from the column and reacted with 4-nitro-*o*-phenylenediamine. The absorbance was measured at 375 nm after removal of excess reagent. The method has a detection limit of 15 μg ascorbic acid/g of sample, and dehydroascorbic acid can be quantitated after reduction to ascorbic acid with dimercaptoethanol.

Electrochemical Methods

The electro-oxidation of ascorbic acid at an electrode follows an irreversible, EC type of electrode mechanism (95), involving the loss of 2 e − and 2 H + to form dehydroascorbic acid. This product reacts rapidly via an irreversible hydration to form diketogluconic acid. Ascorbic acid has a rapid heterogenous rate constant with many types of electrodes; therefore, it is not surprising to find many electroanalytical methods. These procedures are based on dynamic electroanalytical techniques (i.e., measurements of i, t, and/or q) and have been available for many years (96).

Lindquist (97) and Soderhjelm and Lindquist (98) applied linear sweep voltammetry at the carbon paste electrode to determine ascorbic acid in fresh fruits, vegetables, multivitamins, and beverages (97, 98). Voltammograms were run in metaphosphoric acid solutions at scan rates of 20 mV/s. The linearity range extended between 10^{-6} and 10^{-3}M. To determine ascorbic acid in the presence of iron(II), it was necessary to add 5-nitro-1,10-phenanthroline. This reagent complexed the iron(II), and the complex had an increased oxidation potential, thus allowing ascorbic acid to be selectively measured. The method was compared with a 2,6-dichloroindophenol titrimetric procedure and yielded lower results due to better selectivity.

Differential pulse voltammetry at the glassy carbon electrode has been applied to the determination of ascorbic acid in multivitamins containing iron(II) (99). This method did not require a complexation agent. The multivitamin preparations were dissolved in aqueous solution and filtered. This solution was then diluted with pH 4 McIlvaine buffer and differential pulse voltammograms were obtained by scanning at 5 mV/s with a pulse modulation of 50 mV. Measurements of peak potential for ascorbic acid were obtained at 230 mV versus SCE, and the linearity of the method ranged between 10^{-6} and 10^{-1}M. Differential pulse voltammetry has also been used to determine ascorbic acid in vivo from the striatum of rats and guinea pigs at an electrochemically pretreated electrode (100). A graphite/epoxy electrode was able to differentiate between ascorbic acid and dopamine (101). This procedure has a linearity range for ascorbic acid of 5×10^{-4} and 2×10^{-3}M in the presence of 2×10^{-5}M dopamine.

Mason and coworkers have used amperometric detection of ascorbic acid at a tubular graphite electrode in a flow system (102). The limiting current was monitored at 0.75 V versus SCE and was linear over the concentration range of 10^{-6} to 10^{-3}M. The method is suitable for determining ascorbic acid in multivitamin preparations and is more specific than iodometric titration. Although the method involves manual injection of the samples into a flowing stream (25–30

samples/h), it could be readily automated using recent advances in automated chromatographic sample processors.

Recently, Strohl and Curran (103) used flow injection analysis with a reticulated vitreous carbon flow-through electrode to illustrate the applicability of the technique to determine ascorbic acid in ascorbic acid tablets. This procedure had a detection limit of 0.4 ng/injection. Flow injection analysis has also been used to determine the diffusion coefficient of ascorbic acid (104).

In constant-current coulometric methods used for determination of this vitamin, a reagent is generated in situ at an electrode, which then reacts with ascorbic acid. The amount of current per unit time used to obtain the end point is directly proportional to the original ascorbic acid concentration. Marsh and coworkers used electrogenerated Br_2 to determine the vitamin in ascorbic acid tablets (105). The relative standard deviation of this method was 2%.

Moros and coworkers (106) described an automated, iodine-base coulometric method and found it to be equivalent to the USP method. This method can analyze 25 samples/4 h and only requires 1.5 h of constant operator time. The accuracy and precision of the method were ± 0.3%. Another iodometric, constant-current coulometric method has been published, with accuracies within 0.9% and precision of 0.15% (107). Other coulometric methods involve constant potential coulometric analysis of ascorbic acid with I_2 (108) and coulometric determination of the vitamin with electrogenerated octacyanomolybdate (109).

Enzymatic Methods

Spectroscopic.—Earlier enzymatic methods using ascorbic acid oxidase (EC.1.10.3.3) relied on isolation of the enzyme from plant material. Few practical methods for determination of ascorbate have been reported, because the enzyme was not commercially available. Lee and Dawson (110) presented an in-depth report on the isolation and characterization of ascorbate oxidase. An enzymatic procedure for quantitation of ascorbic acid in fruit juices has been reported (111) and is based on the difference in absorbance before and after incubation. The method response was linear between 1 and 10 μg/mL, with an average recovery of 98 ± 0.8%. The optimal experimental parameters for application of this procedure to plant and animal foods are pH 5.5 for sensitivity and enzyme stability and 30 min incubation at 30°C (112). This procedure gave results comparable to those using 2,6-dichloroindophenol or 2,4-dinitrophenylhydrazine.

Recently, Liu and coworkers have used commercially available ascorbic acid oxidase to develop a suitable method for clinical serum or plasma samples (113). In this procedure, ascorbic acid is specifically quantitated by pretreating one of a pair of replicate samples with enzyme. Both samples are reacted with Fe(III) and the complexometric reagent 2,4,6-tris(2 pyridyl)-*S*-triazine. The difference in the absorbance (593 nm) of these samples is proportional to the original ascorbic acid concentration. The method was linear between 0.01 and 0.10 mg/mL and had a precision of 2.8%. The major advantages of the method were (1) endogenous compounds present in biological samples do not interfere, (2) deproteinization of samples is unnecesary, and (3) the procedure is complete within 25 min with routine clinical laboratory equipment.

The enzyme horseradish peroxidase has also been used for the determination of ascorbic acid. Roe and Bruemmor have described a kinetic procedure based on the lag time produced between ascorbic acid, *p*-phenylenediamine, and horseradish peroxidase (114). In this procedure, the enzyme converts *p*-

phenylenediamine to its colored oxidized form. Ascorbic acid rapidly reacts with the oxidized form, converting it back to phenylenediamine. The time required for production of a stable color (of oxidized p-phenylenediamine) is proportional to the original amount of ascorbic acid. This method has been applied successfully to the quality assurance for the labeling compliance of fruit juices. Another variation substitutes guaiacol or homovanilic acid for p-phenylenediamine and is suitable for trace determination in plant extracts (115).

Electrochemical.—Many workers used electrochemical techniques to monitor the depletion of oxygen during the following reaction.

$$AA + 1/2\ O_2 \xrightarrow[\text{Oxidase}]{\text{Ascorbate}} DHAA + H_2O$$
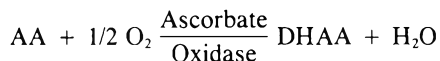
Marchesini and coworkers used a Clark electrode to monitor the depletion of oxygen for this reaction (116). Their procedure was applied to ascorbic acid analysis in fresh and canned spinach. Dehydroascorbic acid was quantitatable only after reduction to ascorbic acid with homocysteine, and the method gave results comparable to the 2,6-dichloroindophenol and 2-4 dinitrophenylhydrazine procedures. Posadka and Macholan coated a Clark oxygen electrode with a thin coat of insolubilized ascorbate oxidase (117). This produced linear results between 10 and 100 mg. Of 35 compounds tested, only chlorogenic acid interfered. Ascorbate oxidase has also been immobilized on collagen and mounted on a Clark electrode (118). The linearity of the method ranged from 5×10^{-5} and 5×10^{-4}M, and the precision was better than 2.3% over 35 successive assays. The ascorbate-selective electrode is stable for 3 weeks and has been applied to food analysis.

An excellent paper has described coupling ascorbate oxidase with chronoamperometry (119) to determine ascorbic acid in brain homogenate. In this procedure, a chronoamperometric response for total oxidizable compounds in brain homogenate was first determined. A known amount of ascorbate oxidase was added, and the total of oxidizable compounds (minus ascorbic acid) was monitored by chronoamperometry. Ascorbic acid content was determined as the difference between the 2 readings. The differential technique requires 1 s or less for each measurement, and the entire assay is completed in a few minutes. The method's specificity has been corroborated by liquid chromatography, and the method has a precision of 2.6%.

Chromatographic Methods

Because many of the spectroscopic and electrochemical methods that have been discussed cannot distinguish ascorbic acid, dehydroascorbic (DHAA), d-isoascorbic (IAA) (also known as erythorbic acid), or other oxidizable compounds, it is not unusual that separation methods have been used to increase selectivity. The following chromatographic techniques will be discussed in some detail: paper and thin layer chromatography, gas chromatography, and liquid chromatography.

Paper and Thin Layer Chromatography.—Numerous paper chromatographic methods have been reported for the qualitative and quantitative determination of ascorbic acid. De Ritter has reviewed many of these procedures (120). Initial paper chromatographic methods were concerned with identifying suitable visualizing reagents or optimal elution solvents. Partridge reported the first qualitative separation of ascorbic and dehydroascorbic acids (121). Cold ammoniacal AgNO₃ was used to visualize the chromatographic zone. Dehydroascorbic acid was detected after heating.

Mapson and Partridge proposed a method to separate ascorbic and isoascorbic acids, using Whatman No. 1 filter paper with an acidified KCN (potassium cyanide) developing solvent (122). Phenol–acetic acid has been used in combination with metaphosphoric acid-impregnated paper (123) or glass fiber filter paper impregnated with silica gel (124) to eliminate the hazardous solvent combination. The method reported by Mitchell and Patterson (123) was used to quantitate human urinary ascorbic acid excretion (125). In this method, ascorbic and erythorbic acids were measured using a 2-6-dichloroindophenol method after isolating each component from the filter paper. Recoveries of 101% and 108% were reported for rat urine and cress seedlings (126). This procedure involved a preliminary separation using a butanolic solvent followed by a second separation with a phenolic solvent. Ascorbic acid was eluted and measured via a DCIP procedure with a recovery of 85% at the 0.5 mg/mL level. A 2,4-diphenylhydrazine method has also been reported using separation by paper chromatography (127).

Thin layer chromatography has been more useful than paper chromatography for quantitation of ascorbic acid. For example, Saari and coworkers studied oxidation of ascorbic acid using a ¹⁴C-labeled compound and densitometric and radioautographic detection (128). The separation system resolved ascorbic and dehydroascorbic acids and an additional compound that was presumed to be 2,3-diketogulonic acid. A densitometric detection method has also been used to measure ascorbic acid in pharmaceutical syrup formulations containing vitamins A, B₁, C, and D₃ (129). Lyle and Tehrani (130) described another exotic thin layer chromatographic method that involves the pyrolysis of thin layer zones directly into a flame ionization gas chromatograph. This method was linear over the range of 0.2–3.0 μg ascorbic acid.

Thin layer chromatographic determinations of ascorbic acid should attract renewed interest in the coming years because of advances in high performance thin layer chromatography (131). The major advantages of this separative technique include increased sensitivity and resolution, ability to run several chromatograms in parallel, and capability of using densitometric and fluorimetric detection.

Gas Chromatography.—The discovery and application of gas chromatography to trace organic analysis produced dramatic improvements in sensitivity and selectivity. The thermal conductivity and flame ionization detectors were the most universal detectors. Unfortunately, polar compounds (e.g., ascorbic acid) were not easily analyzed by this technique.

Sweeley and coworkers (132) reported the first gas chromatographic analysis of ascorbic acid. Trimethylsilylether derivatives were synthesized to increase volatility. The derivatization reaction was carried out in a mixture of hexamethyldisilazane and trimethylchlorosilane (2 + 1) in pyridine. This basic procedure has been modified for the derivatization of ascorbic acid (133–136). The rate of derivatization and elimination of voluminous ammonium chloride precipitation has been investigated. N-Trimethylsilylacetamide (137) or 7,O-bis(trimethylsilyl)acetamide (138) were appropriate reagents. Because ascorbic acid may be derivatized at any one of its 4 hydroxyl groups, Vecchi and Kaiser (137) characterized the silyl derivative of ascorbic acid with gas chromatography–mass spectrometry and concluded that the tetra-trimethylsilylether derivative was formed.

A GC procedure for quantitation of ascorbic acid, pyridoxine, and nicotinamide in multivitamin preparations has been reported by Sennello and Argoudelis (138). This method gave results similar to the USP procedure and has a linearity range

between 50 and 400 μg and a recovery of 98%. Another GC procedure has a linearity range of 0.7–7.0 mg ascorbic acid in orange drink (135). Schlack has also published a method suitable for food products and pharmaceuticals (136); ascorbic acid was precipitated as a lead salt, derivatized, and detected via flame ionization. Linearity was observed between 0.1 and 1.0 mg, and recovery was 100.1%.

Liquid Chromatography.—Liquid chromatography (LC) has been used extensively for ascorbic acid analysis. This technique combines high selectively and sensitivity with rapid sample analysis and does not require derivatization. A minireview of LC methods used for ascorbic acid has recently appeared (139).

(a) *Electrochemical detection:* The early LC methods for ascorbic acid analysis used electrochemical detection (LCEC). Kissinger et al. (140) used a thin layer amperometric cell to measure ascorbic acid in urine. Diluted urine was directly injected onto a strong anion-exchange column, and ascorbic acid was detected at a carbon paste electrode set at + 0.8 V vs Ag/AgCl. Analyses were complete within 4 min, and high selectivity, reduced sample preparation, and decreased analysis time were reported. Multivitamin preparations, fruit juices, milk products, and urine were analyzed using this method (141, 142). Samples were extracted with 3% metaphosphoric acid in 8% acetic acid, and the supernate was diluted with cold perchloric acid (0.05M) before injection. Liquid chromatography with amperometric detection has also been used to measure ascorbic acid in human serum, plasma, and leucocytes (143). Samples were deproteinized with 6% trichloroacetic acid, and ascorbic acid concentrations were quantitatable between 2 and 30 μg/mL. A similar method has been reported for urine and plasma (144).

Thrivikraman and coworkers (145) reported a method applicable to mouse, rat, and guinea pig tissues. Sample preparation consisted of homogenizing the tissue, dilution of the supernate, and then separation on an ion-exchange column. Recovery was 102% with a linear range of 15–50 ng/injection. This method provides increased sensitivity and selectivity when compared with ultraviolet detection. Low nanogram levels of ascorbic acid have also been monitored in various vertebrate and invertebrate tissues using LCEC (146). This method was compared with the DNPH and α,α'-dipyridyl methods (147). The LCEC and DNPH methods compared favorably, whereas the α,α'-dipyridyl method gave significantly higher values for many sample types.

Ascorbic acid concentrations in human serum, plasma, and leukocytes have been determined by using reverse phase ion-pair LC with amperometric detection (148). Samples were deproteinized before analysis, and 97–100% recoveries were reported over the range 0.1–5 μg/mL. Many common drugs and metabolites did not interfere in this assay.

Green and Perlman (149) and Grun and Loewus (150) have used cleanup procedures to improve LCEC selectivity. Plasma ultrafiltration was used to remove protein before separation by reverse phase ion-pair LC (149). Recovery was 100%, and advantages include increased sensitivity (due to reduced sample dilution) and increased column life. Grun and Loewus (150) used a C-18 Sep-Pak® cartridge to selectively retain interfering species from extracts of algae and growth media. Recovery was >99%. Various sugars, sugar acids, and lactones did not interfere.

Stillman and Ma (151) studied the use of polarography for the quantitation of ascorbic acid in multivitamin preparations. Vitamins B_1, B_{12}, C, and K_3 were simultaneously measured at +1.5 V. Analyses were complete in 9 min; however, no detection limits were reported.

An ion-pair LCEC method was used to measure ascorbic and erythorbic acids in orange juice, rat plasma, urine, liver, and brain tissues (152). Numerous counter ions were investigated, and decylamine gave the best separation. The method was highly specific, and no interferences were observed.

(b) *Spectroscopic detection:* Ultraviolet detection has been used to monitor ascorbic acid after separation by ion-exchange chromatography. Williams and coworkers (153) determined ascorbic acid at 254 nm in orange juice, and the method was specific for ascorbic acid. Another method has described the quantitation of ascorbic acid in small volumes of aqueous humor (154). Aqueous humor (2 μL) was diluted 5-fold, directly injected onto an ion-exchange column, and detected at 254 nm. This method gave results comparable to the Roe and Kuether method. Liebes and coworkers (155) reported the analyses for ascorbic acid in human lymphocyte extracts. Their method also agreed with that of Roe and Kuether. Floridi and coworkers (156) described a method for determining ascorbic acid in food products. Linearity ranged from 5 to 30 μg/mL, and analyses were completed in 20 min. Recently, Ashoor and coworkers (157) desribed a method for fruit juices and vegetables.

Reverse phase LC was used to investigate the urinary excretion profile of ascorbic acid in human subjects (158). These results agreed with the USP titrimetric method. Ascorbic acid was determined in citrus juices with a similar method that gave results comparable to the DCIP method (159). Linearity was observed from 50 to 400 μg/mL. The analysis of the vitamin in a variety of fruit juices and vegetables has been described by Watada (160). Recoveries of > 96% were reported, and the method was linear up to 7 μg ascorbic acid.

Sood et al. (161) described the first ion-pair LC method for determining ascorbic acid in foods and multivitamin products. Several pairing agents were evaluated, and tridecylammonium formate (1 mM) provided the best results. Quantitation at 254 nm gave detection limits of 5 μg/mL, and their method was in excellent agreement with the USP method. The method has been modified and used to measure ascorbic acid levels in potatoes and potato products (162). These authors reported detection limits of 0.02–1.125 μg. A method suitable for the routine analysis of bulk pharmaceutical vitamin-mineral formulations has recently appeared (163). Ascorbic acid levels of 500–5000 μg/mL extract were measurable. The absolute purity of ascorbic acid standards has been determined by reverse phase ion-pair LC coupled with conventional stoichiometric analyses (164). Plots of peak height versus the volume of standardized iodine solution added were linear. The titration end point was determined by extrapolation in zero peak height. Sample purity was calculated by using conventional titrimetric equations. This method offers enhanced selectivity and sensitivity as well as reduced sample requirements over conventional stoichiometric methods.

Bui-Nguyen (165) first reported the separation of ascorbic and isoascorbic acids by LC that used a weak anion-exchange column with detection at 265 nm. These acids were detected in various fruit juices with a similar method (166), and detection limits of 20 ng for each acid were reported. Arakowa and coworkers (167) have also described a procedure for analyzing the acids. Ascorbic acid levels of 500 ng/mL were measured at 254 nm, with a total analysis time of 15 min. DHAA and DHIAA could also be indirectly measured following reduction with H_2S. This method was used to measure ascorbic acid and IAA in rat liver, adrenals, spleen, heart, and kidney tissues (168). Tissues were homogenized and the supernate was diluted and injected onto an LC column. No

interferences were observed, and recoveries of 108% and 107% were reported for the 2 acids, respectively.

The simultaneous determination of dehydroascorbic and ascorbic acids has been unsuccessful, because DHAA is electrochemically inactive or does not absorb UV light at the same wavelengths as ascorbic acid. Nevertheless, several approaches for simultaneously determining ascorbic acid and DHAA have been reported. A combination of UV and refractive index detectors has been used to measure these acids in orange juice and urine (169). Isoascorbic, dehydroascorbic, diketogulonic, and diketogluconic acids were also detected with this method. A simple procedure that uses dual UV detectors to quantitate ascorbic acid and DHAA in food (170), biological and pharmaceutical samples (171), or guinea pig tissue has been reported (172). Filtered liquid samples or aqueous extracts of solid samples were directly injected and ascorbic acid and DHAA were detected at 254 nm and 210 nm, respectively. This method was modified to determine ascorbic acid and dehydroascorbic acids in fruits and vegetables (173). Detection limits were 50 ng and 100 ng, and recoveries of 93% and 92% were reported for the 2 acids, respectively. Both compounds have also been determined in guinea pig, monkey, and human plasma (174) and orange juice (175) with dual UV detector methods. In both procedures, the selectivity of the quinoxaline derivatization reaction was used to enhance the UV absorptivity of DHAA. Ascorbic acid was quantitated by electrochemical (174) and ultraviolet (175) detection in these procedures.

Dennison and coworkers (176) reported a single wavelength method in which ascorbic acid was determined directly, and DHAA was estimated as the difference between ascorbic acid and total ascorbic acid at 244 nm. Total ascorbic acid was determined following quantitative reduction of DHAA with D,L-homocysteine. The acids were measured in a variety of beverages, and analyses were made over the range 10–100 μg/mL. Luminescence detection has been investigated for the direct determination of these acids in ascorbic acid tablets (177, 178). A lucigenin-base-reductant system was the basis for the postcolumn reaction of the acids, and conditions were optimized for maximum ascorbic acid emission. Ascorbic acid and DHAA concentrations of 5–800 μg/mL and 10–100 μg/mL were quantifiable, respectively.

Derivatization procedures have been used to increase the sensitivity of LC methods. Garcia-Castineiras and coworkers (179) used the 2,4-dinitrophenylhydrazine method and monitored the resulting bis-(dinitrophenylhydrazone) derivative at 254 nm. Ascorbic acid concentration was taken as the difference between direct sample derivatization (DHAA and DKG) and derivatization following sample oxidation (ascorbic acid, DHAA, and DKG). This method was applied to the analysis of aqueous humor samples, and ascorbic acid levels of 50–1150 pmol (45–1000 μg/mL sample) were measured. Advantages of this method included increased specificity and small sample volume (20 μL). Van Boekel and Meeuwissen (180) also used DPNH derivatization to determine ascorbic acid in beers. Reaction times were reduced to 2 h by heating at 70°C, and recovery of the vitamin was 100%. The method was highly selective, and no interferences were observed during the analysis of 27 different beers. Selectivity for intact ascorbic acid could be introduced by using the dual sample approach of Garcia-Castineiras (179). Recently, a fluorescent assay was used to determine total ascorbic acid in whole blood (181). The assay consisted of derivatization of ascorbic acid to the quinoxaline derivative after oxidation with ascorbic acid oxidase. Recovery from whole blood was 97%, and fluorescence detection (excitation, 255 nm; emission, 475 nm) provided detection limits of about 35 ng total ascorbic acid/mL blood.

Future Directions

The search for a single ideal method to analyze ascorbic acid in food products and biological and pharmaceutical samples may never be successful. Because each sample type has its own special requirements, a multitude of analytical procedures will always appear in the literature. Future methods, however, will always rely on the basic chemical properties of ascorbic acid or on sophistication of instrumentation to develop selective, sensitive methods. In addition to the many suggestions offered in the text, the following avenues for analysis are offered:

Many spectroscopic or electrochemical methods may not be selective for ascorbic acid, so future advances may involve incorporating solid phase extraction columns (e.g., Bond Elute®, Sep-Pak® cartridges) to isolate ascorbic acid. This type of isolation is amenable to robotic automation. Robotics may prove useful in totally automating the semiautomated quinoxaline method of Egberg (87). Future liquid chromatographic methods may rely on using ≤ 3 μm particle size stationary phases or microbore columns for the selective, sensitive determinations of ascorbic acid. LCEC methods will rely on chemically modified electrodes and on advances in stationary phases to further improve selectivity and sensitivity.

> "Fortified with vitamin C
> it's a sign we often see
> is it there just like they say
> or has it oxidized away." (ref. 141)
>
> A sample may be scrutinized
> to see if the vitamin has oxidized
> no matter what the method.
> The analyst will detect it.
> However, the method is only terrific
> if it's specific.

REFERENCES

(1) Kutsky, J. R. (1973) *Handbook of Vitamins and Hormones*, Van Norstrand and Reinhold Co., New York, NY, pp. 71–78
(2) Hashmi, M. H. (1973) *Assay of Vitamins in Parmaceutical Preparations*, J. Wiley and Sons, New York, NY, pp. 286–323
(3) Gilpin, R. K., Pachla, L. A., & Ranweiller, J. R. (1981) *Anal. Chem.* **53**, 142R-159R
(4) Gilpin, R. K., Pachla, L. A., & Ranweiller, J. R. (1983) *Anal. Chem.* **55**, 70R-87R
(5) Sauberlich, H. E., Green, M. D., & Omaye, S. T. (1980) in *Ascorbic Acid: Chemistry, Metabolism and Uses*, P. A. Seib & B. M. Tolbert (Eds), *Advan. Chem. Ser.* **200**, 199–221
(6) McCormick, D. B., & Wright, L. D. (Eds) (1979) *Methods in Enzymology*, Academic Press, New York, NY, pp. 62D, Sec. 1
(7) Cooke, J. R., & Moxon, R. E. D. (1981) in *Vitamin C*, J. N. Counsell & D. H. Horning (Eds), Applied Science Publishers, London, UK, pp. 167–198
(8) Tillmans, J. (1927) *Z. Untersuch Lebensm.* **54**, 33–43
(9) Karayannis, M. I. (1975) *Anal. Chim. Acta* **76**, 121–130
(10) Karayannis, M. I. (1976) *Talanta* **23**, 27–30
(11) Tillmans, J., Hirsch, P., & Jackish, J. (1932) *Z. Untersuch. Lebensm.* **63**, 241–267
(12) Hoffman, M., Bar-Akiva, A., Tenhum, L., & Berkenstadt, Y. (1970) *Anal. Biochem.* **38**, 35–39
(13) Egberg, D. C., Larson, P. A., & Honold, G. R. (1973) *J. Sci. Food Agric.* **24**, 789–794

(14) Rawal, V. M., Patel, U. S., & Desai. R. J. (1978) *Ind. J. Med. Res.* **67**, 161–164

(15) Nesset, B. L., Windsor, B. L., Humphrey, R. R., & Callantine, M. R. (1967) *Anal. Biochem.* **19**, 89–94

(16) King. D. R., & Little, J. W. (1970) *J. Oral. Med.* **25**, 107–111

(17) Cameron. D. J. (1975) *Proc. Nutr. Soc.* **34**, 121

(18) Garry, P. J., Owen, G. M., Lashley, D. W., & Ford, P. C. (1974) *Clin. Biochem.* **7**, 131–145

(19) Bates, J. F., Hughes, R. E., & Hurley, R. J. (1972) *Arch. Oral Biol.* **17**, 1017–1020

(20) Koch, P., Sidloi. M., & Tonks. D. B. (1980) *Clin. Biochem.* **13**, 73–77

(21) Sauberlich. H., Goad, W. C., Skala, J. H., & Waring. P. P. (1976) *Clin. Chem.* **22**, 105–110

(22) Muller-Molot, W., de Vries, U., Bourgeois, C. F., & Mainguy, P. R. (1975) *Z. Lebensm. Unters. Forsch.* **159**, 47–51

(23) Gorbach, G., Henke, J., & Heical. I. (1968) *Mikrochim. Acta* 886–893

(24) Hiromi, K., Fujimori, H., Yamaguchi-Ito, J., Nakatani. H., Ohnishi. M., & Tonomura. B. (1977) *Chem. Lett.* 1333–1336

(25) Obata, H., Tokuyama, T., Nitta, Y., Takagi, M., & Hiromi, K. (1979) *Agric. Biol. Chem.* **43**, 2191–2192

(26) Hiromi, K., Kuwamoto, C., & Ohnishi. M. (1980) *Anal. Biochem.* **100**, 421–426

(27) Koupparis, M. A., Walczak, K. M. & Malmstadt, H. V. (1980) *J. Auto. Chem.* **2**, 66–75

(28) Burger, N., & Kara-Gasparec, V. (1973) *Talanta* **20**, 782–785

(29) Aly. M. M. (1979) *Anal. Chim. Acta* **106**, 379–383

(30) Pelizzeti, E., Mentasti, E., & Pramauro. E. (1976) *Inorg. Chem.* **15**, 2898–2900

(31) Pelizzeti, E., Mentasti, E., & Pramauro. E. (1978) *Inorg. Chem.* **17**, 1181–1186

(32) Zannoni, V., Lynch, M., Goldstein, S., & Sato, P. (1974) *Biochem. Med.* **11**, 41–48

(33) Okamura. M. (1980) *Clin. Chim. Acta* **103**, 259–268

(34) Okamura. M. (1981) *Clin. Chim. Acta* **115** 393–403

(35) Lloyd, J. V., Davis, P. S., & Lander, H. (1969) *J. Clin. Path.* **22**, 453–457

(36) Day, B. R., Williams, D. R., & Marsh, C. A. (1979) *Clin. Biochem.* **12**, 22–26

(37) Thompsen J. C., & Mottola. H. A. (1984) *Anal. Chem.* **56**, 755–757

(38) Jaselskis, B., & Nelapaty, J. (1972) *Anal. Chem.* **44**, 379–381

(39) Butts, W. C., & Mulvihill, H. J. (1975) *Clin. Chem.* **21**, 1493–1497

(40) McGown, E. L., Rusnak, M. G., Lewis, C. M., & Tillotson, J. A. (1982) *Anal. Biochem.* **119**, 55–61

(41) Arakawa, N., Tsutsumi, K., Sanceda, N., Kurata, T., & Inagaki. C. (1981) *Agric. Biol. Chem.* **45**, 1289–1290

(42) Shieh, H., & Sweet, T. R. (1979) *Anal. Biochem.* **96**, 1–5

(43) Eldaway, M. A., Tawfik, A. S., & Elshabouri. S. R. (1975) *Anal. Chem.* **47**, 461–465

(44) Kamangar. T., Fawzi, A. B., & Maghssoudi, R. H. (1977) *J. Assoc. Off. Anal. Chem.* **60**, 528–530

(45) Hammam, A. S. (1976) *J. Appl. Chem. Biotechnol.* **26**, 611–617

(46) Burgess, A. E., & Ottaway, J. M. (1972) *Analyst* **97**, 357–362

(47) Abdel-Salam, M. A., Abdel-Hamid, M. E., & Abdel-Hady Elsayed, M. (1982) *Sci. Pharm.* **50**, 11–16

(48) Samios, D. N., Karayannis. M. I., & Hadjiioannou. T. P. (1977) *Z. Physik. Chem. Neue. Folge.* **104**, 189–198

(49) White, V. R., & Fitzgerald, J. M. (1972) *Anal. Chem.* **44**, 1267–1269

(50) Hassan, S. S., Abd El Fattah, M. M., & Zaki, M. T. M. (1975) *Z. Anal. Chem.* **277**, 369–371

(51) Berek, J., & Berka, A. (1975) *Anal. Lett.* **8**, 57–61

(52) Kyaw, A. (1978) *Clin. Chim. Acta* **86**, 153–157

(53) Elnenaey, E. S., & Soliman, R. (1979) *Talanta* **26**, 1164–1166

(54) Bajaj, K. L., & Kaur, G. (1981) *Analyst* **106**, 117–120

(55) Jagota, S. K., & Dani, H. M. (1982) *Anal. Biochem.* **127**, 178–182

(56) Roe, J. H., & Kuether, C. A. (1943) *J. Biol. Chem.* **147**, 399–407

(57) Zloch, A., Cerven, J., & Ginter, E. (1971) *Anal. Biochem.* **43**, 99–106

(58) Zloch, Z. (1975) *Mikrochim. Acta* I, 213–217

(59) Terada, M., Watanabe, Y., Kunitomo, M., & Hayashi, E. (1978) *Anal. Biochem.* **84**, 604–608

(60) Beljaars. P. R., Horrocks. W. V. S., & Rondags. T. M. M. (1974) *J. Assoc. Off. Anal. Chem.* **57**, 65–67

(61) Schmidt. K., & Holfelder. E. (1975) *Z. Lebensm. Unters. Forsch.* **157**, 217–220

(62) Navon, A., & Levinson, H. Z. (1974) *Z. Natur. Teil.* **29**, 777–780

(63) Toothill. J., Thompson, S. Y., & Hill, W. B. (1970) *J. Dairy Res.* **37**, 521–528

(64) Friberg, U., Lohmander, S., & Carlsson, G. (1968) *Acta Chem. Scand.* **22**, 3037–3039

(65) Hegenauer, J., & Saltman, P. (1972) *J. Chromatogr.* **74**, 133–137

(66) Aeschbacher, H. V., & Brown, R. G. (1972) *Clin. Chem.* **18**, 965–967

(67) Attwood, E. C., Robey, E. D., Ross, J., Bradley, F., & Kramer, J. J. (1974) *Clin. Chim. Acta* **54**, 95–105

(68) Pelletier, O. (1968) *J. Lab. Clin. Med.* **72**, 674–679

(69) Pelletier, O., & Brassard, R. (1973) *Advan. Autom. Anal.* 73–80

(70) Pelletier, O., & Brassard, R. (1975) *J. Assoc. Off. Anal. Chem.* **58**, 104–109

(71) Pelletier, O., & Brassard, R. (1977) *J. Food Sci.* **42**, 1471–1477

(72) Behrens, W. A., & Madere, R. (1979) *Anal. Biochem.* **92**, 510–516

(73) Wahba, N., Yassa, D. A. & Labib, R. D. (1974) *Analyst* **99**, 397

(74) Schmall, M., Pifer, C. W., & Wollish, E. G. (1953) *Anal. Chem.* **25**, 1486–1490

(75) Schmall, M., Pifer, C. W., Wolish, E. G., Buschinsky, R., & Gainer, H. (1954) *Anal. Chem.* **26**, 1520–1521

(76) Wilson, S., & Guillan, R. A. (1969) *Clin. Chem.* **15**, 282–291

(77) Smith. J. A. (1972) *J. Endocrinol.* **55**, 461–462

(78) Geller, M., Weber, O. W. A., & Senkowski. B. Z. (1969) *J. Pharm. Sci.* **58**, 477–480

(79) Crossland, I. (1960) *Acta Chem. Scand.* **14**, 805–813

(80) Weeks, C. E., & Deutsch, M. (1965) *J. Assoc. Off. Anal. Chem.* **48**, 1245–1248

(81) Hashmi, M. H., Shahid, M. A., Akhtar, M. A., & Chughtai, N. A. (1973) *Microchim. Acta* 901–906

(82) Deutsch, M. J., & Weeks, C. E. (1965) *J. Assoc. Off. Agric. Chem.* **48**, 1248–1256

(83) Deutsch, M. J. (1967) *J. Assoc. Off. Anal. Chem.* **50**, 798–805

(84) Kirk, J. R., & Ting, N. (1975) *J. Food. Sci.* **40**, 463–466

(85) Roy, R. A., Conneta, A., & Saltpeter, J. (1976) *J. Assoc. Off. Anal. Chem.* **59**, 1244–1250

(86) Kirk, J. R. (1977) *J. Assoc. Off. Anal. Chem.* **60**, 1234–1237

(87) Egberg, D. C., Potter, R. H., & Heroff, J. C. (1977) *J. Assoc. Off. Anal. Chem.* **60**, 126–131

(88) Dunmire, D. L., Reese, J. D., Bryan, R., & Seegers, M. (1973) *J. Assoc. Off. Anal. Chem.* **62**, 648-652

(89) *Official Methods of Analysis* (1975) 12th Ed., AOAC, Arlington, VA, secs **43.051–43.055; 43.056–43.062**

(90) DeVries, J. W. (1983) *J. Assoc. Off. Anal. Chem.* **66**, 1371–1376

(91) Deutsch, M. J. (1984) *J. Assoc. Offic. Anal. Chem.* **67**, 380

(92) Szepesi. G. (1973) *Z. Anal. Chem.* **265**, 334–336

(93) Bourgeois, C. F., & Mainguy, P. R. (1974) *Int. J. Vit. Nutr. Res.* **44**, 70–84

(94) Bourgeois, C. F., Czornomaz, A. M., George, P., Belliot, J. P., Mainguy,. P. R., & Watier. B. (1975) *Analusis* **3**, 540–548

(95) Perone, S. P., & Keeflow, W. J. (1966) *Anal. Chem.* **38**, 1760–1763

(96) Heyrovsky, J., & Zuman, P. (1968) *Practical Polarography*, Academic Press, New York, NY, p. 102

(97) Lindquist. J. (1975) *Analyst* **100**, 339–348

(98) Soderhjelm, P., & Lindquist. J. (1976) *Acta Pharm. Suec.* **13**, 201–212

(99) Ballantine. J., & Woolfson, A. D. (1980) *J. Pharm. Pharmacol.* **32**, 353–356

(100) Buda, M., Gonon, F., Cespuglio. R., Jouvet, M., and Pujol, J. F., (1980) *C. R. Acad. Sc. Paris* **290**, 431–434

(101) Falat, L., & Cheng, H. Y. (1982) *Anal. Chem.* **54**, 2108–2111

(102) Mason. W. D., Gardner. T. D., & Stewart, J. J. (1972) *J. Pharm. Sci.* **61**, 1301–1303

(103) Strohl, A. N., & Curran, D. J. (1979) *Anal. Chem.* **51**, 1045–1049

(104) Gerhardt, G., & Adams, R. N. (1982) *Anal. Chem.* **54**, 2618–2620

(105) Marsh, D. G., Jacobs, D. L., & Deening, H. (1973) *J. Chem. Ed.* **50**, 626–628

(106) Moros. S. A., Hamilton, C. M., Heveran. J. E., Donahue. J. J., & Oiveri-Vigh, S. (1975) *J. Pharm. Sci.* **64**, 1229–1231

(107) Edholm. L. E. (1976) *Talanta* **23**, 709–713

(108) Karlsson, R. (1975) *Talanta* **22**, 989–993

(109) Cardova-Drellana, R., & Lucena-Conde, X. (1977) *Talanta* **24**, 124–125

(110) Lee, M. H., & Dawson, C. R. (1979) *Methods in Enzymology* **62**, 30–39

(111) Tono, T., & Fujita, S. (1981) *Agric. Biol. Chem.* **45**, 2947–2949

(112) Tono, T., & Fujita, S. (1982) *Agric. Biol. Chem.* **46**, 2953–2959

(113) Liu, T. Z., Chin, N., Kiser, M. D., & Bigler, W. N. (1982) *Clin. Chem.* **28**, 2225–2228

(114) Roe, B., & Bruemmor, J. H. (1975) *Proc. Florida St. Hort. Soc.* **87**, 210–213

(115) Celardin, F., Cusillo, F. J., & Greppin, H. (1982) *J. Biochem. Biophys. Meth.* **6**, 89–93

(116) Marchesini, A., Montuori, F., Muffato, D., & Maestri, D. (1974) *J. Food Sci.* **39**, 568–571

(117) Posadka, P., & Macholan, L. (1979) *Collection Czechoslov Chem. Commun.* **44**, 3395–3404

(118) Matsumoto, K., Yamada, K., & Osajima, Y. (1981) *Anal. Chem.* **53**, 1974–1979

(119) Schenk, J. O., Miller, E., & Adams, R. N. (1982) *Anal. Chem.* **54**, 1452–1454

(120) De Ritter, E. (1965) *J. Assoc. Off. Agric. Chem.* **48**, 985–991

(121) Partridge, S. M. (1948) *Biochem. J.* **42**, 238–248

(122) Mapson, L. W., & Partridge, S. M. (1947) *Nature (London)* **164**, 479–480

(123) Mitchell, L. C., & Patterson, W. I. (1953) *J. Assoc. Off. Agric. Chem.* **36**, 1127–1130

(124) Hornig, D. (1972) *J. Chromatogr.* **71**, 169–170

(125) Kadin, H., & Oscada, M. (1959) *J. Agric. Food Chem.* **7**, 358–360

(126) Chen, Y. T., Isherwood, F. A., & Mapson, L. W. (1953) *Biochem. J.* **55**, 821–823

(127) Miki, T., Kikuchi, N., & Sahashi, Y. (1962) *J. Vitaminol.* **8**, 279–285

(128) Saari, J. C., Baker, E. M., & Sauberlich, H. E. (1967) *Anal. Biochem.* **18**, 173–177

(129) Kouimtzis, Th. A., & Papadazannis, I. N. (1979) *Mikrochim. Acta* **1**, 145–149

(130) Lyle, S. J., & Tehrani, M. S. (1982) *Res. Dev. Topics* 311–313

(131) Fenimore, D. C., & Davis, C. M. (1981) *Anal Chem.* **53**, 252A–266A

(132) Sweeley, C. C., Bentley, R., Makita, M., & Wells, W. W. (1963) *J. Am. Chem. Soc.* **85**, 2497–2507

(133) Dalgliesh, C. E., Horning, E. C., Horning, M. G., Knox, K. L., & Yarger, K. (1966) *Biochem. J.* **101**, 792–810

(134) Allison, J. H., & Stewart, M. A. (1971) *Anal. Biochem.* **43**, 401–409

(135) Koeppen, B. H. (1973) *S. Afr. J. Sci.* **69**, 376–377

(136) Schlack, J. E. (1974) *J. Assoc. Off. Agric. Chem.* **57**, 1346–1348

(137) Vecchi, M., & Kaiser, K. (1967) *J. Chromatogr.* **26**, 22–29

(138) Sennello, L. T., & Argoudelis, C. J. (1969) *Anal. Chem.* **41**, 171–173

(139) Thompson, J. N. (1982) *Trace Anal.* **2**, 1–67

(140) Kissinger, P. T., Felice, L. J., Riggin, R. M., Pachla, L. A., & Wenke, D. C. (1974) *Clin. Chem.* **20**, 992–997

(141) Pachla, L. A., & Kissinger, P. T. (1976) *Anal. Chem.* **48**, 364–367

(142) Pachla, L. A., & Kissinger, P. T. (1979) *Methods in Enzymology* **62**, 15–24

(143) Tsao, C. S., & Salimi, S. L. (1981) *J. Chromatogr.* **224**, 477–480

(144) Mason, W. D., Amick, E. N., & Heft, W. (1980) *Anal. Lett.* **13**(B10), 817–824

(145) Thrivikraman, K. V., Refshauge, C., & Adams, R. N. (1974) *Life Sci.* **15**, 1335–1342

(146) Carr, R. S., & Neff, J. M. (1980) *Anal. Chem.* **52**, 2428–2430

(147) Carr, R. S., Bally, M. B., Thomas, P., & Neff, J. M. (1983) *Anal. Chem.* **52**, 2428–2430

(148) Lee, W., Hamernyik, P., Hutchinson, M., Raisys, V. A., & Labbe, R. F. (1982) *Clin. Chem.* **28**, 2165–2169

(149) Green, D. J., & Perlman, R. L. (1980) *Clin. Chem.* **26**, 796–797

(150) Grun, M., & Loewus, F. A. (1983) *Anal. Biochem.* **130**, 191–198

(151) Stillman, R., & Ma, T. S. (1974) *Mikrochim. Acta* **1974**, 641–648

(152) Tsao, C. S., & Salimi, S. L. (1982) *J. Chromatogr.* **245**, 355–358

(153) Williams, R. C., Baker, D. R., & Schmit, J. A. (1973) *J. Chromatogr. Sci.* **11**, 618–624

(154) Lam, K. W., & Lee, P. F. (1975) *Investig. Ophthal.* **14**, 947–950

(155) Liebes, L. F., Kuo, S., Krigel, R., Pelle, E., & Silber, R. (1981) *Anal. Biochem.* **118**, 53–57

(156) Floridi, A., Coli, R., Fidanza, A. A., Bourgeois, C. F., & Wiggins, R. A. (1982) *Internat. J. Vit. Res.* **52**, 193–196

(157) Ashoor, S. H., Monte, W. C., & Weltey, J. (1984) *J. Assoc. Off. Anal. Chem.* **67**, 78–80

(158) Wagner, E. S., Lindley, B., & Coffin, R. D. (1979) *J. Chromatogr.* **163**, 225–229

(159) Carnevale, J. (1980) *Food Chem. Austr.* **32**, 302–305

(160) Watada, A. E. (1982) *HortScience* **17**, 334–335

(161) Sood, S. P., Sartori, L. E., Wittmer, D. P., & Haney, W. G. (1976) *Anal. Chem.* **48**, 796–798

(162) Augustin, J., Beck, C., & Marousek, G. I. (1981) *J. Food Sci.* **46**, 312–316

(163) Jenkins, C. (1982) *Pharm. Technol.* March, 53–65

(164) Tsau, J., & Poole, J. W. (1982) *Int. J. Pharm.* **12**, 185–197

(165) Bui-Nguyen, M. H. (1980) *J. Chromatogr.* **196**, 163–165

(166) Geigert, J., Hirano, D. S., & Neidleman, S. L. (1981) *J. Chromatogr.* **206**, 396–399

(167) Arakowa, N., Otsuka, M., Kurata, T., & Inagaki, C. (1981) *J. Nutr. Sci. Vitaminol.* **27**, 1–7

(168) Otsuka, M., Kurata, T., Suzuki, E., Arakowa, N., & Inagaki, C. (1981) *J. Nutr. Sci. Vitaminol.* **27**, 9–15

(169) Doner, L. W., & Hicks, K. B. (1981) *Anal. Biochem.* **115**, 225–230

(170) Finley, J. W., & Duang, E. (1981) *J. Chromatogr.* **207**, 449–453

(171) Rose, R. C., & Nahrwold, D. L. (1981) *Anal. Biochem.* **114**, 140–145

(172) Rose, R. C., & Nahrwold, D. L. (1982) *Anal. Biochem.* **123**, 389–393

(173) Wimalasiri, P., & Wills, R. B. H. (1983) *J. Chromatogr.* **256**, 368–371

(174) Baker, J. K., Kapeghian, J., Verlangieri, A. (1983) *J. Liq. Chromatogr.* **6**, 1319–1332

(175) Keating, R. W., & Haddad, P. R. (1982) *J. Chromatogr.* **245**, 249–255

(176) Dennison, D. B., Brawley, T. G., & Hunter, G. L. K. (1981) *J. Agric. Food Chem.* **29**, 927–929

(177) Farber, C. M., Kanengiser, S., Stahl, R., Liebes, L., & Silber, R. (1983) *Anal. Biochem.* **134**, 355–360

(178) Veasey, R. L., & Nieman, T. A. (1980) *J. Chromatogr.* **200**, 153–162

(179) Garcia-Castineiras, S., Bonnet, V. D., Figueroa, R., & Miranda, M. (1981) *J. Liq. Chromatogr.* **4**, 1619–1640

(180) Van Boekel, M. A. J. S., & Meeuwissen, C. A. J. M. (1983) *J. Chromatogr.* **261**, 176–180

(181) Speek, A. J., Schrijver, J., & Schreurs, W. H. P. (1984) *J. Chromatogr.* **305**, 53–60

SEAFOOD TOXINS

Indirect Enzyme-Linked Immunosorbent Assay for Saxitoxin in Shellfish

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An indirect enzyme-linked immunosorbent assay (ELISA) was developed for the detection of saxitoxin (STX). Antibodies against STX were demonstrated in rabbits 5 weeks after immunizing with STX-bovine serum albumin (STX-HCHO-BSA). In the ELISA, STX-HCHO-BSA or polylysine-STX was coated onto the microtiter plate, followed by incubation with standard toxin and anti-STX antibody. The amount of antibody bound to the solid phase was determined by incubation with goat anti-rabbit IgG peroxidase conjugate and a reaction with chromogenic substrate. Competitive indirect ELISA revealed that the antiserum did not cross-react with either carbamoyl-neo-STX-sulfate or tetrodotoxin. The antibodies for STX cross-reacted with decarbamoyl-STX and neo-STX about 56% and 16% as much as they did with STX, respectively. The lower detection limits for STX, decarbamoyl-STX, and neo-STX in this sytem were about 25, 45, and 156 pg per assay, respectively. When STX added to clams or mussels was assayed, the detection limit for STX was about 50–100 ppb, and recoveries were in the range of 86.8–107%.

Saxitoxin is one of the major and most potent in a group of toxins involved in paralytic shellfish poisoning (PSP) (1–3). The toxin is produced predominantly by the dinoflagellate *Gonyaulax catenalla* (4) and is primarily isolated from toxic mussels, clams (2), and other marine animals in waters inhabited by *Gonyaulax*. The poison has no adverse effect on the shellfish using the dinoflagellate as a food source. Human ingestion of toxin-contaminated shellfish has resulted, however, in paralytic poisoning and occasionally death (2). The amount of PSP poison that causes death in humans is estimated to be about 0.5–4 mg (2). The Food and Drug Administration has set the maximum acceptable level for paralytic poison in fresh, frozen, or canned shellfish at no more than 400 mouse units (MU) or about 80 μg/100 g edible portion (2).

Because of the potential health hazard, a quick, sensitive, and specific method is needed to determine the presence of toxins in shellfish. Due to its simplicity, the mouse bioassay (5) has been adopted as an official method for monitoring the poison; however, this method is neither specific nor sensitive, and also requires a continuous supply of mice. About 0.18 μg STX is required to kill a 20 g mouse in 15 min. Other methods, including fluorometric assay (6) and colorimetric techniques (7), also have sensitivity and specificity problems. More recently, a liquid chromatographic method was developed by Sullivan and Iwaoka (8). Although this method can detect low levels of PSP (0.5–25 ng/assay), it requires expensive equipment, and samples must be analyzed one at a time (8, 9).

Because of the highly specific antigen-antibody interaction, several laboratories have attempted to develop an immunoassay for PSP. As early as 1964, Johnson et al. (10) reported an immunoassay involving a hemoagglutination reaction; however, the sensitivity was not high enough for practical application of the immunoassay. Most recently, Carlson and Guire (11) demonstrated a radioimmunoassay system that

permitted detection of less than 1 ng STX, but the antiserum did not react with neo-STX, and, therefore, the antiserum has only limited use. In our laboratory, we have attempted to produce specific antibodies against STX. Among several approaches tested, we found that the antibody produced in rabbits after immunizing with STX conjugated to bovine serum albumin was useful for STX assay. Subsequently, we developed an indirect enzyme-linked immunosorbent assay (ELISA), and details for production, characterization, and the ELISA protocols for determination of STX in clams and mussels are described here.

Experimental

Materials

Purified STX was provided by E. J. Schantz and R. W. Wannemacher, Jr. Decarbamoyl STX was prepared according to the method of Ghazarossian et al. (12, 13). Neo-STX and tetrodotoxin were supplied by R. E. Carlson, and carbamoyl-neo-STX sulfate was provided by H. Schnoes. Bovine serum albumin (BSA, RIA grade), polylysine (mol. wt. 60,000), Tween 20, 2,2-azino-di-3-ethyl-benzthiazoline-6-sulfonate (ABTS), 30% hydrogen peroxide, and goat anti-rabbit IgG peroxidase were purchased from Sigma Chemical Co. (St. Louis, MO). Complete and incomplete Freunds's adjuvant were obtained from Difco Laboratories (Detroit, MI). Gelatin was purchased from Fisher Scientific Co. (Fair Lawn, NJ). Polystyrene microwell plates (NUNC product, Denmark; No. 2-69620) were obtained from Vangard International Inc. (Neptune, NJ). CF-1 mice were purchased from Harlan/ Sprague-Dawley (Madison, WI). Albino rabbits, female, 5 lb size, were purchased from Klubertanz Rabbit Farm (Edgerton, WI) and were tested to be *Pasteurella*-negative before use. All chemicals and organic solvents were reagent grade or better.

The cultivated mussels *(Mytilus recurvus)* and cherrystone clams *(Merceraria mercenaria)*, harvested from the Chesapeake Bay, MD, were purchased from a local seafood store. Clams were kept refrigerated and mussels were kept in crushed ice. Both extracts were tested by mouse bioassay which showed no toxicity. The naturally contaminated scallop (Pecten grandis) samples, supplied by E. J. Schantz who collected them from the Bay of Fundy in 1980, were shucked and kept frozen at $-20°C$ before extraction.

Preparation of Saxitoxin Antigen

The antigen (STX-HCHO-BSA) was prepared according to Johnson et al. (10) with slight modifications. In a typical experiment, 14.25 mg bovine serum albumin in 6 mL 0.1M sodium acetate buffer (pH 4.2) was reacted with 1.5 mg saxitoxin in the presence of 0.081 mL 37% formaldehyde (w/w). The reaction was carried out at room temperature for 72 h and then at 5°C for another 12 h. The reaction mixture was dialyzed against 2 L acetic acid (0.001M) at 5°C for 72 h with 2 changes of the acetic solution during this period to remove residual-free saxitoxin. The molar ratio of STX:BSA was estimated to be about 16:1 by measuring the free amino groups

before and after reaction (14). The same method was used to prepare the STX-polylysine. Succinyl-STX-BSA and decarbamoyl-STX BSA were prepared according to the procedure of Ghazarossian (15).

Immunization Schedule

Three rabbits were immunized with STX antigen, using a multiple site injection method (16). The fur along the back and the proximal limbs of the rabbits was shaved before injection. Two mL of emulsion was made by mixing 500 μg STX-HCHO-BSA in 0.5 mL sterilized saline and 1.5 mL complete Freund's adjuvant. About 30–50 μL emulsion was injected intradermally at each site over the shaved area, and 0.2–0.25 mL emulsion was injected subcutaneously on each shoulder. Each animal received about 30–40 injections. Three or four weeks after the initial immunization, the rabbits were bled on a weekly basis, and the titers were determined. Six to eight weeks after the initial injection, the animals were boosted intramuscularly with another 500 μg antigen in incomplete Freund's adjuvant emulsion prepared by emulsifying 1 volume of antigen with 1 volume of adjuvant. The same schedule (5–6 weeks interval) was used for subsequent booster injections. The antiserum collected was precipitated with ammonium sulfate to a final 33% saturation. The precipitate was redissolved in water to the same original serum volume, dialyzed against 2 L 0.1M PBS overnight, and then lyophilized. The lyophilized, purified antibody was stored at −4°C.

Enzyme-linked Immunosorbent Assay

Titration of antibody titers.—The ELISA protocols are essentially the same as those we described for the analysis of aflatoxin B_1 (17). The optimal dilution of antigen necessary for precoating microtiter plates was determined by the checkerboard test. Fifty μL antigen (STX-HCHO-BSA) at a concentration of 1.6 μg/mL in 0.5M bicarbonate buffer at pH 9.6, was added in each well and incubated overnight at 4°C. The plate was washed with various amounts of washing solution (0.1M sodium phosphate buffered saline containing 0.1% Tween 20, pH 7.5) in the following sequence: twice with 0.1 mL, twice with 0.2 mL, and 3 times with 0.32 mL each well. Three hundred μL 0.1% gelatin in PBS was added to each well to eliminate nonspecific binding by blocking the plastic surface where protein was not bound. After 30 min to 1 h incubation at 37°C, the well was washed 4 times each with 0.32 mL washing solution. Next, 50 μL of various dilutions of antibody, diluted in PBS containing 0.1% BSA, was added to each well. Following 1 h incubation at 37°C, the plate was washed again to remove the free antibody. Fifty μL goat antirabbit IgG peroxidase conjugate (1:500 dilution in PBS containing 0.1% BSA) was added to each well, and the wells were incubated another hour at 37°C. The plate was washed again. The peroxidase substrate (ABTS in citrate buffer, pH 4.0) of 0.1 mL was finally added to each well. After developing the color 20 min at 37°C, the reaction was terminated by adding 0.1 mL hydrofluoric acid–ethylenediamine tetracetic acid stopping reagent. and absorbance at 410 nm was determined by a Dynatech minireader. The antibody titer was defined as the reciprocal of the antiserum dilution that gives an absorbance at 410 nm that is 0.1 unit greater than that of the pre-immune serum.

Competitive indirect ELISA.—Protocols used for titration of antibody titers were slightly modified in this assay. One hundred μL of an appropriate dilution of antigen in 0.05M carbonate buffer, pH 9.6, was coated to the plate. In addition, 50 μL purified STX, other PSP toxins, or unknown samples were incubated together with 50 μL of appropriate dilution of antibody in each well. The amount of bound antibody was determined by adding 0.1 mL goat antirabbit IgG peroxidase conjugate.

Preparation of Samples

The AOAC acid extraction method (5) was used throughout the experiment for extraction of STX from clam and mussel before ELISA. The whole tissue of clam was used for extraction whereas only the dark gland of mussel was used. Mussels were shucked and the dark glands (hepatopancreas) were removed. About 15 mussels gave a total of 5 g dark glands. The samples (50–100 g clam or 5 g dark gland) were homogenized with an equal volume of 0.1N HCl and then boiled 5 min. After cooling to room temperature, the solution was adjusted to pH 4.0–4.5 and then centrifuged to remove the tissue. The acidic extract was adjusted to pH 7.0 and immediately used in the ELISA.

In the recovery experiment, different concentrations of STX diluted in PBS were injected into 6 groups (5 g each group) of dark gland samples. After 30 min, 5 mL 0.1N HCl was added, and the sample was extracted by the procedures described. For clam samples, toxin was added to 50 g of sample during the homogenization step.

Analysis of Samples by Competitive Indirect ELISA

In a preliminary study, the effect of the acid extracts of blank samples on the indirect ELISA was tested. However, we observed significant interference in the ELISA where more than 50 mg sample was used. The interference was minimal when 5 mg/mL of mussel dark gland or 50 mg/mL of clam meat was used. Subsequently, a phosphate-buffered saline solution (0.1M, pH 7.4) containing either blank mussel dark gland or blank clam meat acid extracts, at concentrations of 5 and 50 mg/mL, respectively, was used for preparation of STX standard and sample solutions for ELISA. A naturally contaminated scallop sample was also analyzed for STX by ELISA. A high level of STX was present in this sample, so high dilution was necessary (1 to 10,000 dilution of the acid extract). ELISA was carried out in regular phosphate buffer instead of buffer containing blank samples for this naturally contaminated sample.

Results and Discussion

In the initial studies, we tested several different STX-BSA conjugates for their ability to produce antibodies against STX. using indirect ELISA to monitor the antibody titer. We found that decarbamoyl-STX BSA, succinyl-STX BSA, and STX-HCHO-BSA all elicited antibody against STX. Because the best antibody titers were obtained from rabbits that had been immunized with STX-HCHO-BSA, our efforts were concentrated on this immunogen. Figure 1 shows a typical titration curve for the antibody titer for a rabbit immunized with this antigen. Figure 2 shows results for the average antibody titers of 3 rabbits over a period of 10 weeks. Good antibody titers were obtained from rabbits as early as the 5th week after immunization. Antiserum titer increased considerably after the first booster.

Antibody specificity was determined by a competitive indirect ELISA in which different STX derivatives were present in the assay system to compete the binding of STX coated to the solid phase with the antibody. In this assay, the STX-polylysine conjugate was coated to the plate because this conjugate gave less aggregation after repeated thawing. After incubation with the antibody and different STX derivatives, the rabbit antibody bound to the STX-polylysine solid phase
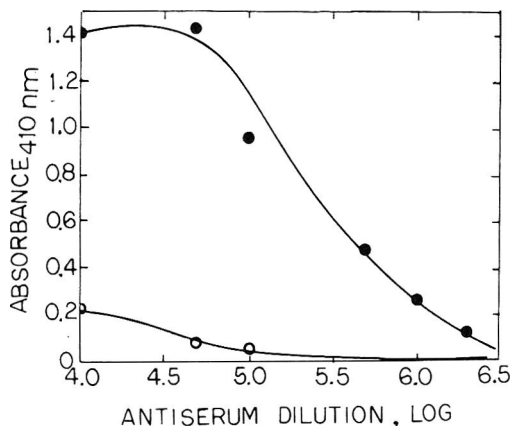
Figure 1. Titration of antibody titer by indirect ELISA. See text for description of procedure. Titer is defined as reciprocal of antiserum dilution that gives an absorbance at 410 nm that is 0.1 unit greater than that of the pre-immune serum. Antisera from rabbits immunized with STX-HCHO-BAS (●); pre-immune serum (○).

Figure 3. Competitive indirect ELISA for STX. See text for description of procedure. Saxitoxin (●), neo-saxitoxin (○), decarbamoyl-saxitoxin (◐), carbamoyl-neo-saxitoxin sulfate (△), and tetrodotoxin (▲).

Figure 2. Average antibody titers for 3 rabbits immunized with STX-HCHO-BSA. Range of antibody titer for bleeding of 3 rabbits is represented by an error bar. Arrow indicates time of booster injection.

Figure 4. Effect of clam and mussel extracts on indirect ELISA of STX. Curves represent data obtained from experiments using STX in PBS buffer. Data from experiments using clam and mussel extracts are shown as bars. STX concentrations for spiking experiments are indicated to left of bars on X-axis. All data represent average of 3 measurements.

was determined by reaction with goat-antirabbit peroxidase complex and enzyme substrate. Results (Figure 3) indicate that the antibody has high affinity for STX. The concentrations that cause 50% inhibition of binding of the antibody to the solid phase antigen (i.e., STX-HCHO-polylysine) by STX, decarbamoyl-STX and neo-STX are 3.2, 50, and 180 ng per assay, respectively. Thus, the antibodies for STX cross-reacted with decarbamoyl-STX and neo-STX only about 56.2% and 16% as much as they did with STX. Carbamoyl neosaxitoxin sulfate and tetrodotoxin showed no cross-reaction with the antibody at the maximal concentration tested.

Carbamoyl neo-STX sulfate did not cross-react with the antibodies whereas neo-STX did, so modification of the structure of the C-11 position may greatly affect the conformation of STX and thus prevent these derivatives from reacting with the antibody. Because the decarbamoyl-STX cross-reacts with the antibodies somewhat, the role of the terminal amide bond in the STX in determining the antibody specificity may not be as important as the side chain in the C-11 position. Additional experiments using other derivatives such as 11-hydroxysaxitoxin sulfate, which is produced by *Gonyaulax tamarensis* and is also a major PSP along the Alantic Coast (3), are needed to verify this hypothesis.

Competitive ELISA results reveal that the present ELISA system can detect 2–10 pg STX (0.1–0.5 ng STX/mL) and

150 pg neo-STX (3 ng/mL) in each assay where 3 standard deviations of the blank response were 5% of binding (Figure 3). It has been reported that the reactivity of neo-STX with anti-saxitoxinol antibody, which was obtained from rabbits immunized with saxitoxinol-BSA conjugate, is less than 1% of those for STX (11). Therefore, the anti-BSA-HCHO-STX antiserum has some advantage over the anti-saxitoxinol antibody, and can be used for monitoring the neo-STX, one of the major toxins in PSP.

Although the indirect ELISA could detect as little as 2 pg STX in each assay when the analysis was carried out with pure STX in buffered solution, high interference was observed when the food samples were analyzed. The problem was particularly serious when whole mussel meat extract was used. Results (Figure 4) indicate only a slight effect on the standard curve when an extract equivalent to 50 mg/mL (or 2.5 mg/assay) of clam meat was used; however, high interference was observed when an extract equivalent to as little as 5 mg/mL (0.25 mg/assay) of mussel meat was used. Therefore, subsequent recovery experiments, including the standards, were carried out in a buffer that contained either blank extract of clam meat (50 mg/mL) or blank mussel dark gland (5 mg/mL) extract. The rationale for using dark gland in the assay of STX in mussel is based on the observation (1) that more than 95% of the PSP is accumulated in this gland, and

Table 1. Recovery (%) of saxitoxin from clam and mussel by ELISA[a]

Toxin added, ppb	Clam			Mussel		
	Rec., % (N)	SD[b]	CV[b]	Rec., % (N)	SD[b]	CV[b]
50	207 (12)	27.3	13.2	95.2 (12)	17.4	18.3
100	103 (20)	15.8	15.3	97.5 (20)	15.8	16.2
200	107 (24)	9.5	8.9	106.5 (12)	9.6	9.0
250	—[c]	—[c]	—[c]	89.6 (8)	8.8	9.8
500	90.8 (24)	11.8	13.0	86.8 (12)	11.4	13.1
1000	105.2 (24)	9.3	8.8	95.4 (14)	19.0	19.9

[a]Saxitoxin was added to whole clam tissue or mussel dark gland. In the assay, either whole tissue (clam) or whole dark gland (mussel) extract was used. In the final calculation, weight of whole mussel was used. In general, dark gland of mussel accounted for about 10% of whole mussel weight.
[b]SD and CV represent standard deviation (%) and coefficient of variation (%), respectively.
[c]Not determined.

that this gland is relatively easy to separate from the meat tissue. Several cleanup protocols were tested, but we felt that adding the blank sample extracts to the assay was the simplest approach in solving the interference problem. Table 1 gives results for the recovery of STX spiked in mussel dark gland and clam. Recoveries were between 86.8 and 107% in the range 50–1000 ppb (on the basis of total tissue weight) and 90.8–105% in the range of 100–1000 ppb STX added to the mussel dark gland and clam samples, respectively. Coefficients of variation for these assays are in the range 9–20%, which is in the range for most ELISAs. The detection limit appears to be in the range of 50–100 ppb where the blank response at 95% confidence was 3 standard deviations.

A naturally contaminated scallop sample was tested by mouse bioassay according to the AOAC method (5) and by the present ELISA. The sample contained 97.8 ± 7.8 μg STX/g sample by ELISA and 112.53 ± 6.53 μg/g by mouse assay. In the bioassay, a series of standard solutions were injected into the same strain of mice under the same experimental conditions where 0.363 μg STX was determined to be 1 mouse unit. Present results indicate that 86.9% of the STX determined by mouse bioassay was detected by ELISA. The lower recovery by ELISA may result from the presence of other toxins in the sample or from problems existing in both assays. Further comparative analyses for additional naturally contaminated samples by both methods as well as testing the cross-reactivity with other PSP toxins, are needed to establish the validity of the assay.

Conclusions

Results obtained from the recent study indicate that antibodies obtained from rabbits immunized with STX-HCHO-BSA are adequate for the analysis of STX in foods, according to ELISA protocols described. Although the antibodies also recognize neo-STX, the ELISA for this toxin is not as sensitive as that for STX. The antibodies, however, could not recognize STX when the OH-group at the C-11 position was esterified by sulfate, i.e., carbamoyl-neo-STX-sulfate. Additional experiments are needed to prepare other STX-protein conjugates for eliciting antibodies that will recognize other PSP toxins.

Acknowledgments

REFERENCES

(1) Baden, D. G. (1983) Int. Rev. Cytol. 82, 99–150
(2) Schantz, E. J. (1979) Pure Appl. Chem. 52, 183–188
(3) Boyer, G. L., Wichmann, C. F., Mosser, J., Schantz, E. J., & Schnoes, H. K. (1979) in Toxic Dinoflagellate Blooms, D. L. Taylor & H. H. Saliger (Eds), Elsevier North Holland, Inc., New York, NY, pp. 373–376
(4) Sommer, H., Whedon, W. F., Kofoid, C. A., & Stohler, R. (1937) Arch. Pathol. 24, 537–559
(5) Official Methods of Analysis (1984) 14th Ed., AOAC, Arlington, VA, secs 18.086–18.092
(6) Bates, H. A., Kostriken, R., & Rapport, H. (1978) J. Agric. Food Chem. 26, 252–254
(7) Gershey, R. M., Nevé, R. A., Musgrave, D. L., & Reichardt, F. B. (1977) J. Fish. Res. Board Can. 34, 559–563
(8) Sullivan, J. J., & Iwaoka, W. T. (1983) J. Assoc. Off. Anal. Chem. 66, 297–303
(9) Sullivan, J. J., Simon, W. G., & Iwaoka, W. T. (1983) J. Food Sci. 48, 1312–1314
(10) Johnson, H. M., Frey, P. A., Angelotti, R., Campbell, J. E., & Lewis, K. H. (1964) Proc. Soc. Exp. Biol. Med. 117, 425–430
(11) Carlson, R. E., & Guire, P. E. (1983) in Symposium on Seafood Toxins, Sept. 1983, American Chemical Society, Washington, DC
(12) Ghazarossian, V. Z., Schantz, E. J., Schnoes, H. K., & Strong, F. M. (1976) Biochem. Biophys. Res. Commun. 68, 776–780
(13) Koehn, F. E., Ghazarossian, V. E., Schantz, E. J., Schnoes, H. K., & Strong, F. M. (1981) Bioorg. Chem. 10, 412–428
(14) Habeeb, A. F. S. A. (1966) Anal. Biochem. 14, 328–336
(15) Ghazarossian, B. E. (1977) Ph.D. thesis, The University of Wisconsin-Madison, Madison, WI
(16) Nieschlag, E., Kley, H. K., & Usadel, K. H. (1975) in Steroid Immunoassay, E. H. D. Cameron, S. G. Hillier, & K. Griffiths (Eds), Alpha Omega Publications, Ltd, Wales, pp. 87–96
(17) Fan, T. S. L., & Chu, F. S. (1984) J. Food Prot. 47, 263–266

EXTRANEOUS MATERIALS

Improved Method for Extraction of Light Filth from 90% Rye Bran Crisp Bread

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A new technique has been developed for the analysis of light filth in a high bran rye product. Compared with the official AOAC method, 44.063, the plates were much easier and less time-consuming to read microscopically (about 8 min compared with about 34 min for the AOAC method). Average recoveries for insect fragments and mouse hairs were 93.4 and 90.8%, respectively, with coefficients of variation (32 samples) of 7.7 and 7.9%, respectively. Average recoveries by the AOAC method were 87.5% for insect fragments and 62.5% for mouse hairs, with coefficients of variation (6 samples) of 7.9 and 14.1%, respectively. A ruggedness test for 7 variables in the extraction indicated that 2 variables caused significant changes in the results.

The current AOAC method (1) for the extraction of light filth from high bran breads is tedious and lengthy. When used to analyze a very high bran content bread, it yields extraction papers containing large amounts of extraneous plant material, which makes counting difficult and causes analyst eye strain and fatigue. A new method, which was developed for the analysis of light filth in a high bran rye product, yielded extraction papers that contained minimal amounts of extraneous plant material. Changes in this method as compared with the AOAC method involved the amount of acid used during acid hydrolysis, the sieve mesh, defatting step, extraction vessel, extraction medium, and extraction oil. A sequestering agent was also added (Table 1).

METHOD

Apparatus

Only glass or metal apparatus should be used, because insect fragments and rodent hairs adhere to plastics.

(a) *Autoclave.*—*(1)* Slow exhaust type: Set slow exhaust to lower pressure from 15 to 0 psi in 15–20 min. *(2)* Non-slow exhaust type: Let cool to 0 psi before opening or venting.

(b) *Trap flask.*—2 L Erlenmeyer.

(c) *Stirring rod with wafer stopper.*—(Entomological Supplies, Inc., Baltimore, MD 21227).

(d) *Sieve.*—Plain weave, USA standard No. 230, 8 in. diameter.

(e) *Rapid flow filter paper.*—32 cm pre-pleated filters (Schleicher and Schuell, Inc., 543 Washington St, Keene, NH 03431) No. 588, or equivalent.

(f) *Rapid flow filter paper cup.*—Center filter paper over bottom of 600 mL beaker. Partially shape paper over bottom of beaker and gently insert beaker into 1 L beaker. Remove 600 mL beaker.

(g) *Ruled filter paper.*—Use smooth, high wet strength, rapid-acting 9 cm filter paper ruled with oil, ethanol, and water-proof lines 5 mm apart (Schleicher and Schuell) No. 8, or equivalent.

(h) *Microscope.*—American Optical (AO) wide field stereomicroscope, or equivalent.

Reagents

(a) *Dilute HCl.*—Dilute 30 mL HCl to 1 L with water.

(b) *Silicone spray.*—Packaging Equipment Slipicone (Dimethylpolysiloxane plus silica aerojel) (Dow Corning Corp., Midland, MI 48640).

(c) *Isopropanol.*—Purified grade.

(d) *Tween 80–40% isopropanol solution.*—To 40 mL polysorbate 80 (I.C.I. United States, Inc., Chemical Research Dept, Wilmington DE 19899) add 210 mL isopropanol–water (40 + 60), mix, and filter.

(e) *Na_4 EDTA solution.*—Dissolve 5 g technical grade tetrasodium ethylene diaminetetraacetate in 150 mL water, add 100 mL isopropanol, mix, and filter.

(f) *Mineral oil.*—Paraffin oil, white, light, 125/135 Saybolt Universal Viscosity (38°), sp. gr. 0.840–0.860 (24°) (Fisher Scientific Co., 711 Forbes Ave, Pittsburgh, PA 15219) No. 0-119, or equivalent.

(g) *Flotation liquid.*—Mixture of mineral oil and *n*-heptane containing <8% toluene (85 + 15).

Procedure

Weigh 50 g sample in 2 L beaker. Break product into 1 in. pieces. Add 1 L dilute HCl. Spray silicone into beaker for ca 1–2 s. Autoclave 30 min at 121°C. Wet sieve with forceful stream of hot (55–70°C) tap water until effluent is clear. Wash sieve retainings to side of sieve and soak with 100 mL isopropanol. Quantitatively transfer sieve retainings with 500 mL isopropanol to preshaped, rapid flow filter paper cup in 1 L beaker. Place beaker on preheated hot plate and boil gently 10 min. Remove cup from beaker, place on Buchner funnel, and aspirate to slow drip. Discard filtrate, replace cup in 1 L beaker, and repeat boiling and aspiration steps twice with 500 mL isopropanol.

Quantitatively transfer filter paper retainings to 2 L Erlenmeyer (trap) flask with 40% isopropanol. Wash filter paper thoroughly. Bring volume of flask to 800 mL with 40% isopropanol. Add magnetic stirring bar (stirring rod clamped at midpoint of flask), and boil gently for 10 min while stirring magnetically. Rinse sides of flasks with 40% isopropanol in wash bottle to prevent material from accumulating and drying on flask. Remove flask from hot plate and place in water bath. Cool flask to room temperature (20–25°C). Add 50 mL flotation liquid down stirring rod, stir magnetically 5 min, and let stand another 5 min. Mix 50 mL Tween 80–40% isopropanol, 50 mL Na_4EDTA solution, and 200 mL 40% isopropanol. Pour mixture slowly down stirring rod, with top of disk or rubber stirring stopper held just below surface of liquid. Mix contents 1 min by gently swirling with stirring rod just beneath surface of liquid. Let flask stand 5 min. Pour 40% isopropanol slowly down stirring rod to bring top of oil layer 1 cm from top of flask. Gently stir plant material at bottom of flask 5 s with stirring rod every 5 min for 20 min. Clamp stirring rod at midpoint of flask and rinse rod with 40% isopropanol. Let flask stand undisturbed 10 min. Trap off into beaker, rinsing neck of flask and rod with 40% isopropanol. Add 35 mL flotation liquid and hand-stir with stirring rod 1 min to disperse oil throughout liquid phase. Pour 40% isopropanol slowly down stirring rod to bring top of oil layer 1 cm from top of flask. Gently stir plant material at bottom of flask 5 s with

Table 1. Differences between the official and the proposed method

Equipment and procedures	AOAC	Proposed
Acid hydrolysis (HCl)	50 mL	30 mL
Sieve mesh	No. 140	No. 230
Defatting procedure	in sieve, isopropanol or ethanol and chloroform	in filter paper cup, isopropanol
Extraction vessel	percolator or Kilborn funnel	trap flask
medium	acid–alcohol	40% isopropanol
oil	mineral oil	flotation liquid
Sequestering agent	none	Tween 80–40% isopropanol and Na$_4$EDTA solution

Table 2. Comparison of official and proposed methods for recovery of light filth from 90% rye bran crisp bread

	AOAC			Proposed		
	Rec., %			Rec., %		
Parameter	Insect frags	Mouse hairs	Time, min	Insect frags	Mouse hairs	Time, min
Range	85–95	50–75	17–45	75–100	75–100	5–14
Average (N)	87.5(6)	62.5(6)	34.3(6)	93.4(32)	90.8(31)[a]	8.3(32)
SD	6.9	8.8	9.3	7.2	7.2	2.3
CV, %	7.9	14.1	27.1	7.7	7.9	27.3

[a]One piece of data rejected as outlier.

Table 3. Ruggedness test results and effect of altered conditions on recovery of light filth

	Boiling time		Flotation liquid			Tween 80–40% Na$_4$EDTA	
Parameter	Defatting, min	Trap flask, min	Amount, mL	Stirring time, min	Standing time, min	Ratio, mL	Standing time, min
Ruggedness test conditions	10(15)[a]	10(15)	50(55)	5(3)	5(10)	50:50(55:45)	5(10)
Change in insect fragment rec., %	4.375	1.875	− 10.625[b]	− 3.125	11.875[c]	− 3.125	− 0.625
Change in mouse hair rec., %	1.25	−2.5	−6.25	2.5	−3.75	2.5	10.0[c]

[a]Number in parentheses = altered condition.
[b]Minus sign denotes that recovery obtained under original test condition was lower.
[c]Significant ($P < 0.05$) by Student's t-test.

stirring rod every 5 min for 20 min. Clamp stirring rod at midpoint of flask and rinse rod with 40% isopropanol. Let flask stand undisturbed 10 min. Trap off into original beaker, rinsing neck of flask and rod with 40% isopropanol. Filter combined trappings onto ruled filter paper and examine under microscope at 30×.

Comparative Study

Thirty-two samples of a commercial 90% rye bran crisp bread were analyzed by the technique described above. In addition, 6 samples of a commercial 90% rye bran crisp bread from the same product lot were analyzed by the official method (1), using 50 g of product each so that both methods would contain the same amount of product. (The official method calls for 225 g of product.)

Ruggedness Test

Sixteen samples (2 for each determination) of a commercial 90% rye bran crisp bread were subjected to a ruggedness test (2).

Spiking

In both the comparative study and the ruggedness test, 20 granary weevil elytral squares (ca 0.6–1 sq. mm) and 20 mouse hairs (ca 1–4 mm) were added to each sample after the bread was broken into ca 1 in. pieces. Previous analysis of a portion of this bread had indicated no contamination from rodent hairs or granary weevil fragments.

Results and Discussion

For insect fragment recovery, the proposed method gave slightly better results than the official method, and precision was about the same (Table 2). Significantly improved recoveries and precision ($P < 0.05$, Student's t-test) for mouse hairs were obtained with the proposed method.

Compared with the official method, reading the plates obtained from the proposed method took much less time. The

AOAC   **PROPOSED**
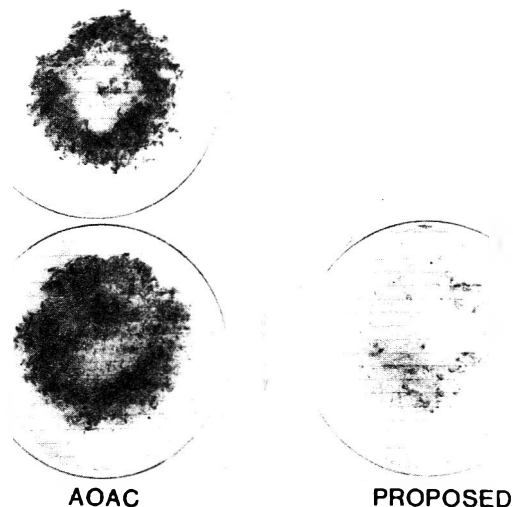
Figure 1. Comparison of plates obtained by official method and proposed method for extraction of light filth from 90% rye bran crisp bread.

2 extraction papers (per 50 g sample) yielded by the official method contained excessive amounts of extraneous plant material (Figure 1) and were very difficult to examine, requiring an average of 34.3 min to read. The one extraction paper (per 50 g sample) yielded by the proposed method contained minimal amounts of extraneous plant material and were very easy to examine, requiring an average of 8.3 min to read (Table 2). Recoveries of mouse hairs by the official method may have been significantly lower because they were obscured by the large amounts of extraneous plant material on the extraction papers.

Analysis of the ruggedness test results (Table 3) indicated a significant decrease for insect fragment recovery ($P < 0.05$, Student's t-test) when the time allowed for standing after

stirring the flotation liquid was increased from 5 to 10 min. Mouse hair recoveries decreased significantly ($P < 0.05$, Student's t-test) when standing time after stirring the Tween 80–40% isopropanol and Na_4EDTA solution mixture was increased from 5 to 10 min. Thus, it is important to adhere closely to these timing steps to obtain maximum recoveries.

Recommendation

For the analysis of a very high bran content bread, filth recoveries, especially for mouse hairs, are higher with the proposed method than with the official method. Because the extraction papers are cleaner, they are easier to read micro-scopically and much time is saved. Analyst eye strain and fatigue are also reduced.

Further work is needed to determine if the proposed method is applicable to lower bran content breads or if further modifications in the method are needed. If satisfactory, the method will be collaboratively studied for possible recommendation for adoption as an official method.

References

(1) *Official Methods of Analysis* (1984) 14th Ed., AOAC. Arlington. VA, sec. **44.063**
(2) Youden. W. J., & Steiner. E. H. (1975) *Statistical Manual of the AOAC*, AOAC. Arlington, VA

DRUG RESIDUES IN ANIMAL TISSUES

Screening Tests for Sulfa Drugs and/or Dinitrobenzamide Coccidiostats and Their Monoamino Metabolites in Chicken Livers

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Two procedures were developed for the simultaneous determination of 0.1 ppm sulfaquinoxaline and sulfadimethoxine, 1.0 ppm Zoalene and nitromide, and/or 0.1 ppm of their reduced coccidiostat metabolities from the same sample of chicken liver. Both methods include blender extraction of 5 g liver with chloroform–ethyl acetate (1 + 1), adsorption of the drugs and metabolites on neutral alumina, and subsequent elution with 0.2M carbonate buffer (pH 11.0). In Method A, all parent drugs and coccidiostat metabolites were partitioned into dichloromethane, following the addition of a small amount of tetrabutylammonium hydroxide (TBAH). The presence of the dinitrobenzamides was confirmed by the formation of a color with TBAH, which occurs when the solvent is concentrated (Zoalene = green; nitromide = red). Sulfa drugs and coccidiostat metabolites were detected by the Bratton-Marshall reaction after thin layer chromatographic (TLC) separation. Method B separates the individual classes by selective extraction techniques. The coccidiostats and their metabolites were extracted from the buffer eluate by ethyl acetate–dichloromethane (3 + 1) before ion pairing: sulfa drugs were extracted with dichloromethane after ion pairing with TBAH. The detection techniques were similar to those described for Method A.

A wide variety of drugs (1) are available for the prevention and control of caecal and intestinal coccidiosis in chickens. Among the drugs are aklomide. 2-chloro-4-nitrobenzamide, and the dinitro-substituted compounds nitromide (3,5-dinitrobenzamide) and Zoalene (3,5-dinitro-*o*-toluamide)—all of which are considered to be potentially toxic to humans because they contain nitro-groups. Although analytical methods are available for detecting and quantitating the individual drugs in chicken tissues (2), the procedures are generally time consuming and cumbersome. As a result, few data have been obtained to determine the extent of the residue occurrence in animal tissues caused by the use of these drugs. The need for a rapid, simple screening procedure for this purpose is evident.

The coccidiostats are readily metabolized (3) in vivo to monoamino compounds, which are included in the violative levels established by the regulatory agencies for chicken livers (4). The procedure for determining Zoalene residues does not determine its metabolites, which require a separate method (5). However, the latter method along with the analytical procedures for aklomide, nitromide, and their metabolites relies on the Bratton-Marshall (B-M) reaction that is widely used for the colorimetric detection of sulfonamides in animal feeds and tissue. Sulfaquinoxaline, and to a lesser extent sulfadimethoxine, are also fed to chickens to prevent coccidiosis and certain microbial diseases. Hence, the use of the B-M reagent creates the potential for the erroneous determination of sulfa drugs in the presence of coccidiostat metabolites in chicken tissues and vice versa. The screening methods presented here were designed to overcome this problem.

METHOD

Reagent and Materials

(a) *Solvents.*—Ethyl acetate, hexane, dichloromethane, methanol, and acetonitrile (Distilled-in-Glass®, Burdick and Jackson Laboratories, Inc., Muskegon, MI 49442). Chloroform, Baker Analyzed reagent (J. T. Baker Chemical Co., Phillipsburg, NJ 08665). *N,N*-Dimethylformamide (Aldrich Chemical Co., Inc., Milwaukee, WI 53233).

(b) *Tetrabutylammonium hydroxide (TBAH)*—40% aqueous solution (Aldrich Chemical Co., Inc.).

(c) *0.2M and 0.5M carbonate buffer.*—pH 11. Prepare from 0.2M and 0.5M solutions of sodium carbonate and sodium bicarbonate.

(d) *Pipet tip.*—5 mL (Rainin Instrument Co., Woburn, MA 01801).

(e) *Neutral alumina.*—Brockman Activity I, 80–200 mesh (Fisher Scientific Co., King of Prussia, PA). Insert 5 mm glass bead into 5 mL pipet tip. Layer glass bead with 0.5 cm sea sand followed by 3 cm bed of neutral alumina—packed firmly by gently tapping top of pipet tip. Add 0.25 cm layer of sea sand. Wash column with three 2 mL portions of CHCl₃–ethyl acetate (1 + 1) before use.

(f) *Sulfa drugs.*—Sulfadimethoxine (Hoffmann-La Roche, Inc., Nutley, NJ 07110); sulfaquinoxaline (Pfaltz and Bauer, Inc., Stamford, CT 06902).

(g) *Aklomide; nitromide; Zoalene.*—Salsbury Laboratories, Charles City, IA 50616.

(h) *3-Amino-5-nitro-o-toluamide (3-ANOT).*—Gift from Dow Chemical USA, Midland, MI 48640. 3-Nitro-5-amino-*o*-toluamide (5-ANOT), 2-chloro-4-aminobenzamide, and 3-amino-5-nitrobenzamide—see acknowledgments.

Apparatus

(a) *Centrifuges.*—Sorvall Superspeed centrifuge, Type SS-1 rotor (Ivan Sorvall, Inc., Norwalk, CT). International Clinical centrifuge, rotor No. 273 (International Equipment Co., Needham Heights, MA 02194).

(b) *Vortex stirrer.*—Super Mixer (Lab-Line Instruments, Inc., Melrose Park, IL 60160).

(c) *Tissue grinder.*—Brinkmann Polytron® homogenizer (Brinkmann Instruments Inc., Westbury, NY 11590).

(d) *Liquid chromatography (LC).*—Altex Model 100A pump (Altex Scientific Inc., Berkeley, CA 94710) connected to Schoeffel Model SF770 Spectroflow variable wavelength detector operated at 254 nm. Altex Model 210 sampling valve with a 50 μL loop. Column—25 cm × 4.6 mm id, 5 μm Alltech C₁₈ (Alltech Assoc., Deerfield, IL 60015). Mobile phases: sulfa drugs—methanol–water–acetic acid (40 + 59.5 + 0.5); coccidiostats—methanol–water (30 + 70); coccidiostat metabolites—acetonitrile–water (10 + 90).

(e) *Thin layer chromatography (TLC).*—2.5 cm × 10.0 cm glass plate with 250 μm layer of silica gel G (Analtech, Newark, DE 19711). Developing solvent—CHCl₃–ethyl acetate–methanol (5 + 5 + 1). Develop plates to 0.5 cm height and dry in forced air oven 1 min at 50°C. Redevelop plates to

height of 1.0 cm, then 4.0 cm, with oven drying between and after attaining final height. Visualize sulfa drugs and cocci-diostat metabolites with Bratton-Marshall (B-M) spray reagent according to procedure previously described (6). R_f values: sulfadimethoxine, 0.74; sulfaquinoxaline, 0.67; 2-chloro-4-aminobenzamide, 0.58; 3-amino-5-nitrobenzamide, 0.55; 3-ANOT, 0.56; and 5-ANOT, 0.45.

Analytical Procedure

Weigh 5 g frozen chicken liver, obtained from cross section of individual organ or from homogenized sample of livers, into 50 mL polypropylene centrifuge tube. Let sample partially thaw. Add 20 mL CHCl$_3$–ethyl acetate (1 + 1) and blend 30 s with Polytron homogenizer at low speed. Centrifuge 5 min at 3000 rpm. Remove aqueous layer with disposable Pasteur pipet and discard. Recover solvent with disposable pipet and filter through 3 cm column of anhydrous sodium sulfate contained in large volume (4 mL) Pasteur pipet plugged at top and bottom with small wad of glass wool. Collect 10 mL filtrate. Pass filtrate through neutral alumina column. Wash column with 7 mL CHCl$_3$. Remove excess CHCl$_3$ from column with air pressure and continue pressure until column dries as evidenced by disappearance of moisture on outside of column. Elute column with 0.2M, pH 11 carbonate buffer, using air pressure if necessary to obtain flow of 1–2 mL/min. Collect first 5 mL eluate in 15 mL screw-cap centrifuge tube. Add 5 mL hexane and shake carefully 1 min, using rocking motion. Centrifuge 2 min at 2500 rpm. Remove hexane with disposable pipet and discard. Proceed to Method A or Method B.

Method A—Simultaneous Detection of Sulfa Drugs and Dinitrobenzamide Coccidiostats and Their Monoamino Metabolites

Add 30 μL TBAH solution to eluate in centrifuge tube and vortex-mix 15 s. Add 10 mL dichloromethane and shake vigorously 2 min, using rocking motion. Centrifuge 2 min at 2500 rpm. Recover dichloromethane as previously described (6). Evaporate solvent in 9 mL screw-cap specimen vial at 40°C under stream of nitrogen to just dryness, noting color of residue. Definite green color indicates presence of Zoalene; red indicates nitromide. Dissolve residue in 0.2 mL methanol and spot 10 μL in 1.0–1.5 μL increments on TLC plates, drying spot with stream of nitrogen between applications. Subject samples to TLC and B-M spray reagent to visualize sulfa drugs and monoamino coccidiostat metabolites.

Method B—Separate Detection of Sulfa Drugs and Dinitrobenzamide Coccidiostats and Their Monoamino Metabolites

(a) *Monoaminobenzamide metabolites.*—Add 1 mL 0.5M, pH 11 buffer and 9 mL ethyl acetate–dichloromethane (3 + 1) to alumina eluate in centrifuge tube. Shake vigorously 2 min, using rocking motion. Centrifuge 2 min at 2500 rpm. Remove ethyl acetate–dichloromethane with disposable Pasteur pipet and evaporate to dryness in 9 mL screw-cap specimen vial at 50°C under stream of nitrogen. Dissolve residue in 0.2 mL methanol. Spot 10 μL solution and subject samples to TLC and B-M visualization reagents as described above.

(b) *Dinitrobenzamide coccidiostats.*—Evaporate TLC methanol solution in (a) above to dryness. Dissolve residue in 0.1 mL DMF. Add 1 drop of TBAH solution and note any color formation—green indicates presence of Zoalene; red indicates nitromide.

(c) *Sulfa drugs.*—Add 30 μL TBAH solution to ethyl acetate–dichloromethane extracted alumina eluate in (a) above.

Mix thoroughly. Add 9 mL dichloromethane and shake vigorously 2 min, using rocking motion. Centrifuge 2 min at 2500 rpm. Recover dichloromethane and evaporate to dryness at 50°C in 9 mL screw-cap specimen vial. Dissolve residue in 0.2 mL methanol. Subject sample to TLC and B-M visualization reagents according to procedure described above.

Recovery Studies

Fresh chicken livers were obtained locally and held frozen at −10°C. Five g samples of individual livers were spiked while frozen with methanol solutions of the drugs and/or metabolites (sulfaquinoxaline and sulfadimethoxine, 0.12 μg/μL; Zoalene and nitromide, 0.4 μg/μL; 3-ANOT and 5-ANOT, 0.18 μg/μL; and 2-chloro-4-aminobenzamide, 0.19 μg/μL). The spiked livers were held frozen for 1 h before extraction. Controls for the spiked samples were obtained from the same liver. Recovery data were determined by LC. To the residues obtained by Method A or B was added 0.4 mL of the appropriate mobile phase. The samples were vortex-mixed 30 s, followed by centrifugation 1 min at 2500 rpm. Fifty μL samples were injected onto the LC column. Recoveries were determined on the basis of peak heights relative to those of known quantities of the drugs and metabolites. TBAH was excluded from recovery studies of the coccidiostats by Method A because the dinitrobenzamides decomposed on evaporation. Difficulties in purifying 3-amino-5-nitrobenzamide prevented carrying out recovery studies on this metabolite. Retention times (min) were sulfadimethoxine, 12.8; sulfaquinoxaline, 15.2; Zoalene, 13.2; nitromide, 14.5; 3-ANOT, 11.9; 5-ANOT, 16.1; and 2-chloro-4-aminobenzamide, 8.5.

Results and Discussion

Preliminary studies revealed that Zoalene, nitromide, and aklomide were readily metabolized in vitro in chicken livers to their monoamino derivatives at room temperature. These observations were, generally, in keeping with previous reports (3, 7). Hence, screening procedures for detecting the coccidiostats in liver tissues must detect the in vivo metabolites and/or those that arise in vitro as the result of the unavoidable time lapse between sacrifice of the birds and freezing of the excised liver tissue. Because the methods of detecting the monoaminobenzamide metabolites are the same as those used for sulfa drugs (i.e., the B-M reaction), the screening procedure must differentiate these 2 classes of drugs. Method A differentiates the coccidiostat metabolites, sulfadimethoxine and sulfaquinoxaline, on the basis of TLC retention values; Method B separates the coccidiostat metabolites from the sulfa drugs before TLC separation and detection procedures by selective extraction techniques.

The parent dinitrobenzamide coccidiostats are detected in both methods by the formation of a color with TBAH at >1.0 ppm (liver basis). Aklomide, a mononitrobenzamide, does not form a color and is not detectable as the parent compound by the screening procedure. In Method A, TBAH acts initially as a counter ion to extract the sulfa drugs from the basic solution with dichloromethane. On evaporation of the solvent, the partitioned TBAH forms the color with the dinitrobenzamide coccidiostats. In actual practice, the concentrated solution takes on the appropriate color when approximately 0.5–1.0 mL solution containing >1.0 ppm coccidiostat remains. In Method B, at relatively high concentrations, the appropriate color is formed with DMF alone, but the color is greatly intensified by the addition of TBAH. In both procedures, control sample extracts exhibit only slight yellow colors in the presence of TBAH.
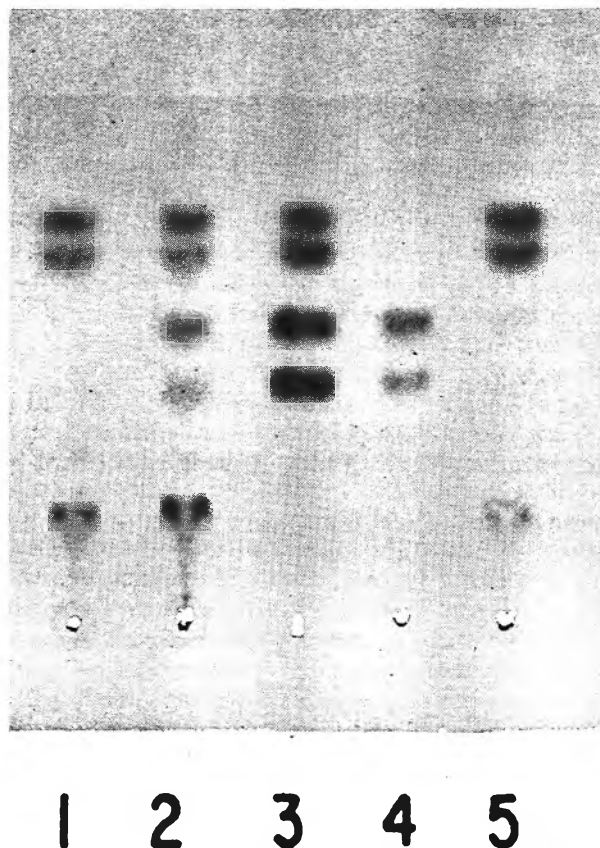
Figure 1. TLC of extracts of chicken livers treated according to Methods A and B. Livers spiked with 0.4 ppm sulfadimethoxine, 0.4 ppm sulfaquinoxaline, and 1.0 ppm Zoalene before analyses. 1) Unabused liver, Method A. 2) Abused liver, Method A. 3) Authentic sulfadimethoxine; sulfaquinoxaline; 3-ANOT; 5-ANOT (decreasing R_f). 4) Abused liver, Method B, coccidiostat fraction. 5) Abused liver, Method B, sulfa fraction. Abused liver—sample thawed and held 30 min at ambient temperature.

Table 1. Recovery (%)[a] of sulfa drugs, dinitrococcidiostats, and coccidiostat metabolites added to chicken livers

Sample	Added, ppm	Method A	Method B
Sulfadimethoxine	0.1, 0.2, 0.4	78.5 ± 4.0	71.0 ± 4.5
Sulfaquinoxaline	0.1, 0.2, 0.4	63.9 ± 5.5	62.3 ± 8.8
Zoalene	1.0	78.2 ± 1.4	79.4 ± 3.1
3-ANOT	0.5	34.4 ± 3.3	61.1 ± 1.2
5-ANOT	0.5	17.2 ± 1.7	38.6 ± 2.7
Nitromide	1.0	60.2 ± 2.6	66.5 ± 5.7
2-Chloro-4-aminobenzamide	0.5	23.6 ± 1.8	36.7 ± 1.5

[a]Mean and standard deviation of 6 determinations at each concentration.

The monoaminobenzamide metabolites, sulfaquinoxaline and sulfadimethoxine, are detected using the B-M spray reagent following TLC. In addition to detecting the Zoalene and nitromide metabolites, the procedures are capable of detecting 0.1 ppm 2-chloro-4-aminobenzamide. In contrast to previous reports (8), studies have shown that a second metabolite, tentatively identified as 5-ANOT on the basis of TLC and LC studies, is formed during the in vitro metabolism of Zoalene. This latter observation aids in differentiating the presence of Zoalene metabolites from the singular metabolites of nitromide and aklomide in liver tissue, despite similar R_f values. The separation of the sulfa drugs and coccidiostat metabolites into their respective classes by Method B is not absolute because approximately 10% of each class of drugs is carried over into the other class. The carryover does not, however, prevent recognition of the class of drugs present, and it can be avoided by additional extractions, if desired.

Figure 1 illustrates the TLC results of Methods A and B on chicken liver samples spiked with sulfadimethoxine, sulfaquinoxaline, and Zoalene. Holding sample 1 at − 10°C before using Method A resulted in the detection of the sulfa drugs. Holding sample 2 at room temperature 30 min before before using Method A showed the additional presence of 3-ANOT and 5-ANOT. Samples 4 and 5, representing the coccidiostat and sulfa fractions obtained by using Method B on an abused liver, demonstrate the separation of classes by this procedure. The irregular (bottom) B-M positive spot in samples 1, 2, and 5 is anthranilic acid, a naturally occurring compound in liver tissues (9). The detection limits for sulfadimethoxine and sulfaquinoxaline have been found to be <0.1 ppm. Although

only these sulfa drugs have been used in this study, others should give the same results.

Table 1 summarizes the drug recoveries obtained by using Methods A and B on chicken livers spiked with varying concentrations of sulfa drugs, coccidiostats, and coccidiostat metabolites. Although the recoveries of the coccidiostat metabolites were low, no interferences were noted. Also, there was no difficulty detecting them at the 0.1 ppm level by TLC, partially as a result of the tight bands obtained. Attempts to improve recoveries of the metabolites by varying solvent systems resulted in lower recoveries and therefore higher detection limits, of the sulfa drugs.

The procedures were applied to livers of white leghorns that were fed a diet containing 0.0125% Zoalene for 5 weeks, and were sacrificed while still on the medicated feed. The livers were placed in dry ice within minutes of sacrificing the chickens. There was no difficulty detecting Zoalene by using either method on these samples. TLC of extracts obtained by either method revealed relatively large amounts of both the 3- and 5-ANOT. In addition, 3 minor in vivo metabolites were present in these extracts, the identities of which are unknown but currently under investigation. The minor metabolites, which may appear by using these methods, do not interfere with assessing the nature of the drug residues. The significance of minor metabolites is revealed in the fact that the 6.0 ppm violative level established for Zoalene in chicken livers (4) includes the parent drug plus the single metabolite, 3-ANOT. This study suggests that setting a violative level for a readily metabolized drug such as Zoalene without the ability to measure all principal metabolites may be unrealistic in determining whether a significant residue problem is present.

In conclusion, an analyst running 4 samples concurrently by Method A can complete the analyses in 2 h: Method B requires an additional 1/2 h for completion. Method B has the advantage of eliminating any doubt as to the class of compounds present in the tissues.

Acknowledgments

REFERENCES

(1) McDougald, L. R., & Reid, W. M. (1983) *Feedstuffs* 55(34), 23–24
(2) Markus, J. R. (1978) *J. Assoc. Off. Anal. Chem.* 61, 1289–1298
(3) Thiegs, B. J., Smith, G. N., & Bevirt, J. L. (1961) *J. Agric. Food Chem.* 9, 201–204

(4) *Tolerances for Residues of New Animal Drugs in Food* 21 Code Fed. Reg. 556 (1981)
(5) Theigs, B. J. (1966) *J. Assoc. Off. Anal. Chem.* **49**, 708–711
(6) Parks, O. W. (1982) *J. Assoc. Off. Anal. Chem.* **65**, 632–634
(7) Zietlow, D. C., Petzold. E. N., George, G. M., & Morrison, J. L. (1969) *J. Agric. Food Chem.* **17**, 1059–1061
(8) Smith, G. N.. Thiegs, B. J., & Ludwig, P. D. (1963) *J. Agric. Food Chem.* **11**, 257–260
(9) Parks, O. W.. & Kimoto, W. I. (1982) *J. Assoc. Off. Anal. Chem.* **65**, 1285–1287

Quantitative Gas Chromatographic–Mass Spectrometric Assay of Five Sulfonamide Residues in Animal Tissue

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A gas chromatographic–mass spectrometric procedure using isotopically labeled internal standards and the technique of selective-ion monitoring was used to assay sulfamethazine, sulfadimethoxine, sulfabromethazine, sulfathiazole, and sulfaquinoxaline in liver and muscle tissue of swine, poultry, and cattle. The data presented clearly demonstrate the applicability of this procedure for confirmation of sulfonamide residues in animal tissue.

With the availability of relatively inexpensive low resolution quadrupole mass spectrometers, mass spectrometry can be routinely used for regulatory purposes (1). In 1981, the authors' laboratory published a gas chromatographic–mass spectrometric (GC/MS) procedure (2) designed to assay sulfamethazine in swine liver and muscle tissue. That procedure was subjected to an AOAC collaborative study (1) and subsequently was adopted official first action. Other food-producing animal species are treated with sulfamethazine and with other sulfonamide drugs; therefore, emphasis was placed on expanding the applicability of the original procedure. This paper details the modifications required to accomplish this expansion of applicability.

METHOD

Apparatus

(a) *Gas chromatograph/mass spectrometer (GC/MS).*—Hewlett Packard Model 5992 low resolution quadrupole GC/MS, or equivalent, equipped with membrane separator.

(b) *Chromatographic column.*—3 ft × 1/8 in. glass, packed with 3% OV-17 on 80–100 mesh Gas-Chrom Q.

(c) *Rotary evaporator.*—Buchi Rotavapor R-110, or equivalent; N-Evap, Model 111 (Organomation Associates, Inc.), or equivalent.

(d) *Diazomethane generator.*—Aldrich Chemical Co. No. Z-10, 159-1.

(e) *Chromatographic column.*—1 × 20 cm (Kontes Glass Co. No. K-420280).

Reagents

(a) *Solvents.*—Acetone, methylene chloride, methanol, chloroform, ethyl ether, hexane, ethyl acetate (all UV grade distilled-in-glass products of Burdick & Jackson Laboratories, Muskegon, MI 49442).

(b) *Sulfonamides, 13C-labeled.*—Sulfmethazine, sulfadimethoxine, sulfathiazole, sulfaquinoxaline, sulfabromethazine (SMZ, SDM, STZ, SQX, SBM) (Kor Isotopes, Cambridge, MA 02142). Isotopic purity was stated to be 90.5% at each of the 6 positions on the phenyl moiety of the molecule.

(c) *Sulfonamides, 12C-labeled.*—SMZ, STZ, SQX (Pfaltz and Bauer, Inc., Stamford, CT 06902); SDM (Hoffmann-La Roche, Inc., Nutley, NJ 07101); SBM (Merck & Co., Inc. Rahway, NJ 07065).

(d) *Pentafluoropropionic anhydride (PFPA).*—Reagent grade (Pierce Chemical Co., Rockford, IL 61105).

(e) *Diethylamine (DEA).*—Reagent grade (Fisher Scientific Co., Fairlawn, NJ 07410).

(f) *Florisil.*—60–100 mesh (Applied Science Laboratories, Inc.), activated 24 h at 130°C.

(g) *Diazomethane.*—Prepare fresh weekly using manufacturer's instructions and the following reagents: N-methyl-N-nitroso-p-toluene sulfonamide (Diazald, Aldrich Chemical Co.. Inc., Milwaukee, WI 53223); anhydrous, reagent grade ethyl ether; diethyleneglycol monoethyl ether (Carbitol); potassium hydroxide pellets.

Note: Prepare diazomethane in hood behind protective shield. Wear gloves to prevent skin contact. Observe caution when handling diazomethane, because it is toxic and, under some conditions, explosive. The recommended conditions and total volume of diazomethane generated minimize instability of compound. Store in freezer.

(h) *Standard solutions.*—13C-labeled multisulfonamide internal standard: 50 μg/mL methanol of SMZ, SDM, SQX, STZ, and SBM. 12C-labeled multisulfonamide fortification standard: 50 μg/mL methanol of SMZ, SDM. SQX, STZ. and SBM.

Sampling

Liver and muscle tissues from swine, turkey, and cattle were analyzed. All samples were fortified before extraction with predetermined amount of labeled (0.1 ppm 13C-sulfonamide) and unlabeled (0.05–0.02 ppm 12C-sulfonamide) drug from stock solutions containing 50 μg/mL.

Extraction and Cleanup

Sulfonamides were extracted from tissue by a modification of Tishler's Method A (3).

Organic extraction and concentration: Place 50 g ground, frozen tissue in 500 mL Virtis flask. Add 100 mL chloroform-acetone (1 + 1) and blend 1 min at slow speed. Decant and filter through 24 cm Whatman 2V fluted paper into 1 L round-bottom flask. Filtrate should be clear and free of particulate matter. Repeat extraction twice. Concentrate combined extracts to oily residue by using rotary evaporator.

Aqueous partition/organic wash: Quantitatively transfer oily residue to 250 mL separatory funnel by using, in order, four 25 mL portions of hexane, two 3 mL portions of acetone, and two 25 mL portions of hexane. Add 10 mL 1N HCl and

gently shake 2 min. Let phases separate and filter aqueous phase through 9 cm Whatman No. 42 paper into 125 mL separatory funnel. Repeat aqueous extraction 3 times using 5 mL portions of 1N HCl. Add 3 mL 10N NaOH and mix; pH should be 12–13. Add 25 mL chloroform and shake 1 min. Let phases separate and discard organic phase. Repeat chloroform wash a second time, again discarding organic phase. Quantitatively transfer aqueous phase to small beaker (100 mL).

Organic partition/concentration: Buffer aqueous phase with 25 mL saturated trisodium citrate and adjust pH to 5.25 ± 0.1, using NaOH or HCl as required. Extract aqueous phase 3 times with 15 mL portions of methylene chloride. Evaporate combined methylene chloride extract to dryness in warm water bath under gentle stream of nitrogen.

Organic wash: Reconstitute residue in 2 mL methanol. Add 4 mL hexane and mix, using vortex mixer. Let phases separate, then aspirate and discard hexane layer (top).

Derivatization

Methylation of N^1 position: Add 1–2 mL freshly prepared saturated ethereal solution of diazomethane. Mix and let stand 5 min. Evaporate to dryness on N-Evap apparatus set at 45°C under gentle stream of nitrogen.

Acylation of N^4 position: Reconstitute methylated extract in 100 μL ethyl acetate and mix. Add 100 μL 20% PFPA in hexane followed by 100 μL 10% DEA in ethyl acetate. Mix and let stand 20–30 min. Evaporate to dryness and reconstitute in 200–400 μL ethyl acetate. Sample is now ready for GC/MS analysis.

Note: An additional cleanup step is required for sulfonamide residues in beef muscle:

Additional column cleanup for beef muscle: Place 2 g 5% water-deactivated Florisil in 1.0 cm × 20.0 cm glass column fitted with coarse glass frit. Prewash column with 10 mL ethyl acetate followed by 10 mL hexane. Add 4 mL hexane to prepared sample, mix, and place on column. Wash column with 10 mL 20% ethyl acetate in hexane. Elute column with 10 mL ethyl acetate, collecting eluate in 15 mL concentrating tube. Evaporate to dryness and reconstitute in 200–400 μL ethyl acetate. Sample is ready for GC/MS analysis.

Preparation of Standards

Add 100 μL ^{13}C-sulfonamide stock solution (50 μg/mL) to each of three 15 mL concentration tubes. Add 50 μL ^{12}C-sulfonamide stock solution (50 μg/mL) to first tube, 100 μL ^{12}C-sulfonamide stock solution to second tube, and 200 μL ^{12}C-sulfonamide stock solution to third tube. Add 1 mL freshly prepared diazomethane to each tube and let stand 5 min. Evaporate contents of each tube to dryness, reconstitute in 100 μL ethyl acetate, and add 20 μL freshly prepared 20% PFPA in hexane, followed by 100 μL 10% DEA in ethyl acetate. Let stand 15–20 min. Gently evaporate to dryness under stream of nitrogen. Reconstitute in 200–400 μL ethyl acetate. Standards prepared in this manner will be equivalent to 0.05, 0.10, and 0.20 ppm sulfonamide tissue concentration.

GC/MS Analysis

GC parameters: Injection port, 260°C; initial column temperature, 210°C; oven program, 5°C/min; final column temperature 260°C, time at final temperature, 10 min; helium flow, 25–30 mL/min. Under these conditions, elution order and approximate retention time (min) for compounds studied were STZ, 4.0; SMZ, 5.0; SDM 7.0; SBM 7.5; and SQX 8.5.

Mass spectrometer parameters (general): Mass spectrometer was operated in selective-ion monitoring mode. Mass

Table 1. SIM files for SBM, SDM, SQX, STZ, and SMZ (200 msec dwell time for each ion)

Sulfonamide	^{12}C-Isotope	^{13}C-Isotope	Fragment
SBM	451*	457	M-65
	452	458	M-64
	238*	244	M-278
SDM	405*	411	M-65
	406	412	M-64
	238	244	M-232
SQX	395*	401	M-65
	396	402	M-64
	238	244	M-222
STZ	350*	256	M-65
	351	357	M-64
	238	244	M-177
SMZ	373*	380	M-65
	374	381	M-64
	238	244	M-200

*Identifies the 6 ions used to establish a survey file.

analyzer and ion optics were set according to parameters determined by instrument Autotune program. Electron multiplier was typically operating in 1800–2000 eV range.

Selective-ion monitoring (SIM) parameters: Selection of SIM parameters depends on specific sulfonamide to be assayed. Instrument used for this study has capability of monitoring 6 ions at any given time. Any combination of 6 ions (within limits of mass analyzer) can be used to establish SIM file. Table 1 identifies SIM files used by this laboratory.

Qualitative identification: Conduct survey run to determine which, if any, sulfonamides are present. Select appropriate SIM file and reassay sample for specific sulfonamide. Criteria for qualitative identification include: (1) coelution of endogenous sulfonamide with internal standard; (2) presence of all 6 ions from appropriate SIM file; (3) ratio of M-64/M-65 fragments for ^{12}C and ^{13}C isotopes should be ± 10% of values determined from standards.

Quantitation: Determine tissue concentration for specific sulfonamide by linear regression. Establish standard curve by analyzing standard solutions containing tissue equivalents of 0.05, 0.10, and 0.20 ppm for each sulfonamide to be assayed. In addition, fortify each standard with 0.10 ppm tissue equivalent of ^{13}C isotopically labeled internal standard of each sulfonamide. Plot ^{12}C (M-65) to ^{13}C (M-65) ion mass ratio against amount of ^{12}C-sulfonamide added to each standard.

Results and Discussion

The electron impact (EI) fragmentation pattern of sulfonamides is well established (4). Although the molecular ion is generally not present, large fragmentation abundances are found that correspond to the molecular ion less 64 (M-64) and 65 (M-65) atomic mass units. These ions correspond to the loss of SO_2 and HSO_2 from the molecular ion. For chromatographic and mass spectrometric purposes, all sulfonamides were derivatized at both the N-1 (methylated) and N-4 (acylated) positions. The additional derivatization step (original sulfamethazine procedure (1) contained only one derivatization step, N-1 methylation) serves 2 purposes: First, it shortens the chromatographic run time and, second, it negates the effect of a background interference (m/z 259) corresponding to the M-65 ^{12}C-fragment of the N-1 methyl derivative of SDM.

The work described in this paper was intended to demonstrate the feasibility of using an inexpensive GC/MS system for screening, confirmation, and quantitation (5). Our approach was to establish, initially, whether a specific sulfonamide was

Table 2. Recovery of sulfonamides from fortified turkey liver

Sulfonamide	N	Added, ppm	Mean found, ppm	SD	CV, %	Qual. ident. requirements[a]		
						a	b	c
SMZ	6	0.00	ND			−	−	−
SMZ	6	0.05	0.048	0.003	6.45	+	+	+
SMZ	6	0.10	0.100	0.003	3.19	+	+	+
SMZ	6	0.20	0.204	0.009	4.24	+	+	+
SDM	6	0.00	ND			−	−	−
SDM	6	0.05	0.050	0.001	1.26	+	+	+
SDM	6	0.10	0.099	0.004	4.0˙	+	+	+
SDM	6	0.20	0.199	0.009	4.59	+	+	+
SQX	6	0.00	ND			−	−	−
SQX	6	0.05	0.049	0.003	6.76	+	+	+
SQX	6	0.10	0.102	0.005	4.97	+	+	+
SQX	6	0.20	0.197	0.006	3.00	+	+	+
STZ	6	0.00	ND			−	−	−
STZ	6	0.05	0.056	0.011	18.90	+	+	+
STZ	6	0.10	0.098	0.011	10.99	+	+	+
STZ	6	0.20	0.197	0.016	8.12	+	+	+

[a] a = coelution, b = 6 ions present, c = M-64/M-65 fragmentation ratio; (+) condition was met, (−) condition was not met.

Table 3. Recovery of sulfonamides from fortified turkey muscle

Sulfonamide	N	Added, ppm	Mean found, ppm	SD	CV, %	Qual. ident. requirements[a]		
						a	b	c
SMZ	6	0.00	ND			−	−	−
SMZ	6	0.05	0.054	0.0001	2.81	+	+	+
SMZ	6	0.10	0.097	0.003	3.18	+	+	+
SMZ	6	0.20	0.200	0.003	4.33	+	+	+
SDM	6	0.00	ND			−	−	−
SDM	6	0.05	0.047	0.008	6.86	+	+	+
SDM	6	0.10	0.099	0.004	4.49	+	+	+
SDM	6	0.20	0.204	0.005	2.67	+	+	+
SQX	6	0.00	ND			−	−	−
SQX	6	0.05	0.051	0.042	8.20	+	+	+
SQX	6	0.10	0.099	0.008	8.56	+	+	+
SQX	6	0.20	0.196	0.006	3.18	+	+	+

[a] See Table 2.

Table 4. Recovery of sulfonamides from fortified swine liver

Sulfonamide	N	Added, ppm	Mean found, ppm	SD	CV, %	Qual. ident. requirements[a]		
						a	b	c
SMZ	3	0.00	ND			−	−	−
SMZ	3	0.05	0.047	0.002	4.46	+	+	+
SMZ	3	0.10	0.098	0.008	10.9	+	+	+
SMZ	3	0.20	0.199	0.002	1.04	+	+	+
SDM	3	0.00	ND			−	−	−
SDM	3	0.05	0.045	0.0065	14.60	+	+	+
SDM	3	0.10	0.098	0.015	15.00	+	+	+
SDM	3	0.20	0.209	0.010	4.81	+	+	+
STZ	3	0.00	ND			−	−	−
STZ	3	0.05	0.057	0.001	2.04	+	+	+
STZ	3	0.10	0.093	0.027	28.60	+	+	+
STZ	3	0.20	0.204	0.032	15.60	+	+	+

[a] See Table 2.

present and, if present, to conduct a more rigorous identification and quantitation. Screening was accomplished by conducting a survey run using a SIM file consisting of the ions identified with an asterisk in Table 1. The survey file contains ions specific and common to each of the 5 sulfonamides studied. When the ion profile of the survey run indicated the presence of a particular sulfonamide, we selected a SIM file more appropriate to that compound (Table 1) and reinjected the sample. Our criteria for qualitative identification, as well as the means by which we arrived at a quantitative value, are stated above.

The relative merits of a qualitative/quantitative analytical response are always open to further discussion. The issue most often associated with our approach is that no absolute recovery is calculated. The values reported in Tables 2–7 are about 100% of what was added to the tissue before conducting the assay. This is not accidental or a "best data" situation. Any analyte loss during sample preparation is automatically compensated for by an equal loss of the internal standard. Absolute recovery from tissue becomes a concern only if insufficient analyte is present; this is easily monitored by comparing the number of counts obtained for the ^{13}C M-65 or M-64 fragment of a standard with the number of counts obtained for the same fragment from a fortified tissue sample. All samples and standards contained 0.1 ppm of the ^{13}C-labeled internal standard.

Table 5. Recovery of sulfonamides from fortified swine muscle

Sulfonamide	N	Added, ppm	Mean found, ppm	SD	CV, %	Qual. ident. requirements[a]		
						a	b	c
SMZ	3	0.00	ND			−	−	−
SMZ	3	0.05	0.047	0.0012	2.47	+	+	+
SMZ	3	0.10	0.091	0.016	17.7	+	+	+
SMZ	3	0.20	0.198	0.020	10.3	+	+	+
SDM	3	0.00	ND			−	−	−
SDM	3	0.05	0.052	0.0065	12.30	+	+	+
SDM	3	0.10	0.098	0.006	6.12	+	+	+
SDM	3	0.20	0.194	0.004	1.95	+	+	+
STZ	3	0.00	ND			−	−	−
STZ	3	0.05	0.046	0.005	11.88	+	+	+
STZ	3	0.10	0.086	0.010	11.46	+	+	+
STZ	3	0.20	0.189	0.016	8.25	+	+	+

[a]See Table 2.

Table 6. Recovery of sulfonamides from fortified beef liver

Sulfonamide	N	Added, ppm	Mean found, ppm	SD	CV, %	Qual. ident. requirements[a]		
						a	b	c
SMZ	3	0.00	ND			−	−	−
SMZ	3	0.05	0.052	0.0047	9.15	+	+	+
SMZ	3	0.10	0.103	0.004	3.9	+	+	+
SMZ	3	0.20	0.197	0.003	1.46	+	+	+
SDM	3	0.00	ND			−	−	−
SDM	3	0.05	0.048	0.0032	6.650	+	+	+
SDM	3	0.10	0.097	0.005	5.21	+	+	+
SDM	3	0.20	0.194	0.007	3.43	+	+	+
STZ	3	0.00	ND			−	−	−
STZ	3	0.05	0.050	0.011	22.70	+	+	+
STZ	3	0.10	0.096	0.011	11.43	+	+	+
STZ	3	0.20	0.188	0.022	11.76	+	+	+
SQX	3	0.00	ND			−	−	−
SQX	3	0.05	0.042	0.002	4.76	+	+	+
SQX	3	0.10	0.100	0.022	21.4	+	+	+
SQX	3	0.20	0.208	0.019	9.17	+	+	+
SBM	3	0.00	ND			−	−	−
SBM	3	0.05	0.048	0.0026	5.51	+	+	+
SBM	3	0.10	0.093	0.0006	0.62	+	+	+
SBM	3	0.20	0.186	0.0067	3.57	+	+	+

[a]See Table 2.

Table 7. Recovery of sulfonamides from fortified beef muscle

Sulfonamide	N	Added, ppm	Mean found, ppm	SD	CV, %	Qual. ident. requirements[a]		
						a	b	c
SMZ	3	0.00	ND			−	−	−
SMZ	3	0.05	0.052	0.0021	4.03	+	+	+
SMZ	3	0.10	0.101	0.0015	2.07	+	+	+
SMZ	3	0.20	0.192	0.0087	4.51	+	+	+
SDM	3	0.00	ND			−	−	−
SDM	3	0.05	0.049	0.0031	6.19	−	−	−
SDM	3	0.10	0.102	0.0004	0.56	+	+	+
SDM	3	0.20	0.196	0.0022	1.47	+	+	+
SBM	3	0.00	ND			−	−	−
SBM	3	0.05	0.048	0.0060	13.01	+	+	+
SBM	3	0.10	0.109	0.0058	6.9	+	+	+
SBM	3	0.20	0.230	0.0073	5.21	+	+	+

[a]See Table 2.

REFERENCES

(1) Malanoski, A. J., Barnes, C. J., & Fazio, T. (1981) J. Assoc. Off. Anal. Chem. **64**, 1386–1391

(2) Suhre, F. B., Simpson, R. M., & Shafer, J. W. (1981) J. Agric. Food Chem. **29**, 727–729

(3) Tishler, F., Sutter, J. L., Bathish, J. N., & Hagman, H. E. (1968) J. Agric. Food Chem. **16**, 50–53

(4) Davis, R., Hurst, D. T., & Taylor, A. R. (1977) J. Appl. Chem. Biotechnol. **27**, 543–548

(5) Code of Federal Regulations (1982) Title 21, Parts 556.640, 556.670, 556.650

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Sensitive Determination of Ethopabate Residues in Chicken Tissues by Liquid Chromatography with Fluorometric Detection

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A liquid chromatographic (LC) method is described for determination of ethopabate residues in chicken tissues. The drug is extracted from tissues with acetonitrile, and the extract is concentrated to 2–3 mL. This aqueous solution is rinsed with ethyl acetate and cleaned up by Florisil column chromatography. LC analysis is carried out on a Zorbax ODS column, and ethopabate is quantitated by using a fluorometric detector set at 306 nm (excitation) and 350 nm (emission). Recoveries of ethopabate added to chicken tissues at levels of 0.01 and 0.05 ppm were 87.8 and 92.7%, respectively. The detection limit was 100 pg for ethopabate standard, and 0.5 ppb in chicken tissues.

Ethopabate, 4-acetamido-2-ethoxybenzoic acid methyl ester, is used as a coccidiostat with amprolium in poultry feeds. According to Japanese regulations (1), the medicated feed must be withdrawn 7 days before slaughter to prevent any residues of ethopabate in the tissues. There is, therefore, a need to monitor residues of ethopabate in chicken tissues at the low levels associated with human health concerns.

Gas chromatographic (GC) methods have been developed for determining ethopabate in feeds (2, 3) and chicken tissues (4, 5). These methods are quite tedious and require considerable time for derivatization of ethopabate before GC measurement (3–5). Liquid chromatography (LC) with UV detection has been used to determine ethopabate in feeds (6), and chicken tissues (7). Because UV detectors are not sufficiently sensitive (6, 7) to monitor residual amounts of ethopabate in tissues, an improved method is necessary.

This paper describes a simple, sensitive method for determination of ethopabate in chicken tissues by LC with fluorometric detection at levels as low as 0.5 ppb. Sample background does not interfere.

METHOD

Reagents

Use analytical reagent grade chemicals and deionized water unless otherwise specified.

(a) *Solvents*—Acetonitrile, ethyl acetate, and *n*-hexane (Wako Pure Chemical Industry Ltd, Osaka, Japan).

(b) *Anhydrous sodium sulfate.*—Wako Pure Chemical Industry Ltd.

(c) *Triethanolamine (TEA).*—Wako Pure Chemical Industry Ltd.

(d) *Florisil.*—60–80 mesh; use as received (Floridin Co.).

(e) *LC elution solvent.*—Acetonitrile–0.01M KH_2PO_4–triethanolamine (40 + 59 + 1) acidified to pH 4.0 with orthophosphoric acid.

(f) *Cleanup column mobile phase.*—Ethyl acetate–*n*-hexane (35 + 65).

(g) *Ethopabate standard solution.*—Prepare stock solution at 100 μg/mL, using 10 mg ethopabate (Dainippon Seiyaku Co., Ltd, Tokyo, Japan; Merck and Co.) in 100 mL acetonitrile. Use 2 mL stock solution to prepare 2 μg/mL intermediate solution. Prepare 0.2 μg/mL working standard solution in acetonitrile, using 10 mL intermediate solution.

(h) *Internal standard solution.*—Dissolve 10.0 mg β-naphthol (Wako Pure Chemical Industry Ltd) in 100 mL acetonitrile. Dilute this solution with acetonitrile to prepare solution of 0.2 μg β-naphthol/mL.

Apparatus

(a) *Liquid chromatograph.*—Shimadzu LC-3A equipped with Shimadzu RF-530 spectrofluorometer and Shimadzu CTO-2A column oven. Chromatographic conditions: flow rate, 1.5 mL/min; temperature, 50°C; detection, 306 nm excitation and 350 nm emission.

(b) *Chromatographic column.*—Zorbax ODS stainless steel, 250 mm × 4.6 mm id (Dupont Co., Analytical Instruments Div., Westwood, NJ 07675).

(c) *High-speed homogenizer.*—Ultra-Turrax T 18 (Janke & Kunkel GmbH & Co., Switzerland).

(d) *Centrifuge.*—Model H-100-F (Kokusan Enshinki Co., Ltd, Tokyo, Japan).

Extraction and Cleanup

Accurately weigh 20 g minced tissue, homogenize twice for 3 min at maximum speed with 50 mL acetonitrile, and then centrifuge 10 min at 3000 rpm. Filter through cotton and concentrate to 2–3 mL under vacuum on rotary evaporator at 40°C. Add 2.0 g sodium chloride and 50 mL ethyl acetate, mix well 3 min, and transfer upper phase to 200 mL flask. Re-extract with another 50 mL ethyl acetate and combine extracts. Add 20 g anhydrous sodium sulfate and let mixture stand 30 min. Filter through cotton and evaporate to dryness under vacuum on rotary evaporator at 40°C. Dissolve residue in 1 mL ethyl acetate–*n*-hexane (1 + 9) and apply to Florisil column. Wash column with 50 mL ethyl acetate–*n*-hexane (1 + 9) and elute ethopabate with 50 mL ethyl acetate–*n*-hexane (35 + 65). Evaporate eluate to dryness under vacuum on rotary evaporator at 40°C and dissolve residue in 0.5 mL internal standard solution (h).

Analysis and Calculation

Inject 10 μL working standard and sample solution into liquid chromatograph, measure peak height ratios to height of internal standard. Calculate concentration by following formula:

$$\text{Ethopabate, ppm} = Rs / Rw \times Cw \times 0.025$$

where Rw and Rs = peak height ratios in working standard and sample solutions injected, respectively; and Cw = concentration of ethopabate in working standard, μg/mL.

Results and Discussion

The fluorescence characteristics of ethopabate were studied using acetonitrile–0.01M KH_2PO_4–TEA (40 + 59 + 1) acidified with orthophosphoric acid to pH 4 as LC mobile phase. Ethopabate shows fluorescence maxima at 306 nm excitation and 350 nm emission, as shown in Figure 1.

The optimal LC operating conditions were studied by varying the mobile phase composition, column temperature, and flow rate. Under the LC conditions selected for detection, as
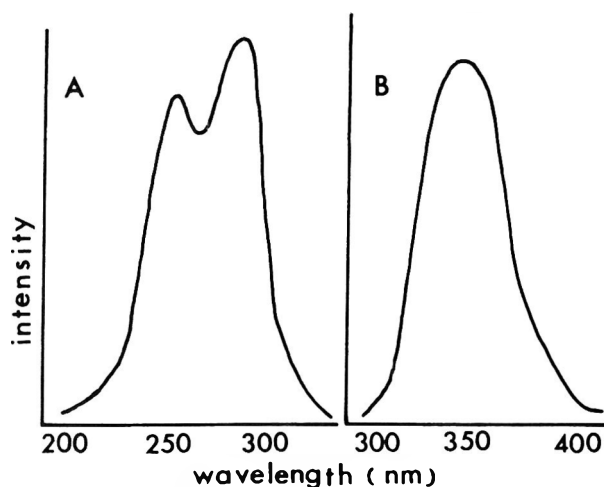
Figure 1.　Fluorescence spectra of 100 μg ethopabate/mL mobile phase. A; excitation spectrum at 350 nm emission; B; emission spectrum at 306 nm excitation.
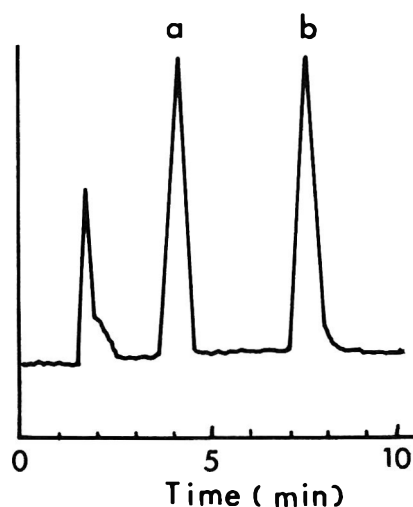
Figure 2.　Typical chromatogram of ethopabate-spiked commercial chicken tissue. a; ethopabate, 0.5 ng; b; β-naphthol, 1 ng. See text for chromatographic conditions.

shown in *Method*, ethopabate is well separated from the solvent front and other interfering components.

A calibration curve was prepared over the range 0.05–2.0 ng ethopabate. The ratios of the peak heights of ethopabate to internal standard plotted against concentration of ethopabate gave a straight line. β-Naphthol was selected as the internal standard because the fluorescence spectra were similar, and the retention time was sufficiently longer than that of ethopabate.

The Florisil column was used to remove other co-extractives that might irreversibly adsorb on the LC analytical column and interfere in the measurement of ethopabate on the chromatogram. Ethopabate was retained when 1 mL of ethopabate in ethyl acetate–n-hexane (1 + 9) was applied to the Florisil column. Interferences were removed with 50 mL ethyl acetate–n-hexane (1 + 9), and then ethopabate was quantitatively eluted from the column with 50 mL ethyl acetate–n-hexane (35 + 65). Figure 2 shows a typical chromatogram for a sample of commercial chicken tissues spiked at the 2.5 ppb level; there were no interfering peaks in the chromatogram. Recovery studies were performed by adding 0.2 and 1.0 μg ethopabate to 20 g minced chicken tissues. These recoveries (Table 1) are satisfactory for residue analysis and substantiate the validity of the method. The utility of the method was demonstrated by its application to commercial chicken tissues.

No ethopabate was detected in 10 commercial chicken tissues by the present method. Determination of ethopabate added to commercial chicken tissues showed that this method is applicable at levels as low as 0.5 ppb. The detection limit for ethopabate standard was 100 pg.

Table 1.　Recovery of ethopabate added to chicken tissues

Added,[a] μg	Found, μg	Rec., %
0.2	0.178	89.4
	0.186	93.1
	0.163	81.6
	0.179	89.8
	0.169	84.9
Av.		87.8
SD		1.43
CV, %		1.63
1.0	0.964	96.4
	0.901	90.1
	0.936	93.6
	0.906	90.6
	0.926	92.6
Av.		92.7
SD		0.03
CV, %		0.03

[a] μg ethopabate added to 20 g chicken tissue.

REFERENCES

(1) Takabatake, E. (1981) *Eisei Kagaku* **27**, 127–143
(2) Smallidge, R. L., Jr (1978) *J. Assoc. Off. Anal. Chem.* **61**, 561–563
(3) Handy, P. R., & Holzer, F. J. (1970) *J. Assoc. Off. Anal. Chem.* **53**, 461–464
(4) Okada, T. (1981) *Shokuhin Eiseigaku Zasshi* **22**, 279–284
(5) Nose, N., Hoshino, Y., Yamada, F., Kikuchi, Y., & Kawauchi, S. (1981) *Shokuhin Eiseigaku Zasshi* **22**, 508–512
(6) Schronk, L. R., Colvin, B. M., Hanks, A. R., & Bushway, R. J. (1977) *J. Assoc. Off. Anal. Chem.* **60**, 1048–1050
(7) Nakazawa, H., Takabatake, E., Hino, S., & Mtema, C. A. (1983) *Bunseki Kagaku* **32**, 179–183

Determination of Neomycin in Animal Tissues, Using Ion-Pair Liquid Chromatography with Fluorometric Detection

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A liquid chromatographic (LC) method is described for the determination of neomycin in animal tissues. Tissues are homogenized in 0.2M potassium phosphate buffer (pH 8.0); the homogenate is centrifuged, and the supernate is heated to precipitate the protein. The heat-deproteinated extract is acidified to pH 3.5–4 and directly analyzed by LC. The LC method consists of an ion-pairing mobile phase, a reverse phase ODS column, post-column derivatization with *o*-phthalaldehyde reagent, and fluorometric detection. The LC method uses paromomycin as an internal standard, and separates neomycin from streptomycin or dihydrostreptomycin because they have different retention times. The LC column separates neomycin in 25 min; the detection limit is about 3.5 ng neomycin. The overall recovery of neomycin from kidney tissues spiked at 1–30 ppm was 96% with a 9.0% coefficient of variation. The method was also applied to muscle tissue.

Neomycin, an aminoglycoside, is classified as a broad-spectrum antibiotic because it inhibits the growth of both gram-positive and gram-negative bacteria (1). Its interaction with other drugs as well as other antibiotics has been reviewed (2). Neomycin is toxic to both the auditory branch of the eighth cranial nerve (3) and nephrons of the kidneys (4) when given parenterally. The toxicity of aminoglycoside antibiotics in general has been reviewed by Clark (5).

In the veterinary profession, antimicrobial drugs including aminoglycosides are used not only for the treatment of animal diseases, but also subtherapeutically for preventing disease and increasing the efficiency of feed utilization (6). Because of their metabolic similarities, neomycin, streptomycin, and dihydrostreptomycin are considered collectively when administered to food-producing animals (6). These aminoglycosides are excreted primarily by the kidney and are concentrated only by the kidney and not the liver (6). Neomycin residues were found in kidney tissues for 28–30 days after oral dosing of animals (6, 7). Neomycin residues were also found in the kidneys of chicks 4 weeks after intramuscular treatment, but no residues were detected after the fifth week (8). Therefore, the use of neomycin in food-producing animals may result in neomycin residues in food derived from these animals.

Numerous chemical and physical methods have been reported for the determination of neomycin. These methods include thin layer (9), gas (10), ion-exchange (11), and liquid chromatography (LC) (12, 13). None of these methods have been used, however, to detect neomycin in biological matrices. Traditionally, microbiological methods have been employed for the detection of residues of neomycin and other aminoglycosides in edible products of animal origin (10, 14–16); however, these methods lack speed and specificity and are inaccurate (17). They do not distinguish neomycin from streptomycin and dihydrostreptomycin, and standardization among laboratories is difficult. The microbiological techniques have little or no potential for detection and quantitation of metabolites of the parent drug. LC is used increasingly as a method for the determination of antibiotics in biological fluids (18), but its application to the determination of residues in edible tissues has only recently been reported (19–21).

This paper reports the development of isolation and LC methods for the detection of neomycin in tissues of food-producing animals.

Experimental

Apparatus

(a) *Liquid chromatograph.*—Waters Associates (Milford, MA) Model 6000A solvent delivery system, Model 730 systems controller, Model 720 data module, Model 710B Wisp autosampler, and Perkin-Elmer (Norwalk, CT) Model 650S fluorescence detector set at 340 nm excitation and 455 nm emission. Slits were set at 5 and 10 nm for excitation and emission, respectively. Detector sensitivity setting was 10.

(b) *LC columns.*—Supelcosil LC-8-DB and LC-18-DB, both 15 cm × 4.6 mm and 5 μm particle size (Supelco, Inc., Bellefonte, PA); Spherisorb ODS-2, 25 cm × 4.6 mm, 5 μm particle size (R. E. Gourley Co., Laurel, MD); μBondapak/ C$_{18}$, 30 cm × 3.9 mm, 10 μm (Waters Associates); and guard columns: 10 μm LiChrosorb RP-18, 3 cm × 4.6 mm (Brownlee Labs, Santa Clara, CA), and 5 μm Supelco LC-8-DB Supelguard (2 cm × 4.6 mm).

(c) *Post-column reaction system.*—Kratos Model URS 050 (Kratos Analytical Instruments, Westwood, NJ).

(d) *Centrifuge.*—IEC Model DPR-6000 (Damon/IEC Division, Needham Heights, MA) with rotor No. 269, set at 4°C, and polypropylene centrifuge tubes with plug-type screw caps (Corning Glass Works, Corning, NY).

(e) *Homogenizer.*—Tissumizer with metal miniprobe (Tekmar Co., Cincinnati, OH).

(f) *Liquid scintillation counter.*—Beckman LS-250 (Beckman Instruments, Inc., Fullerton, CA) set at 10°C; Aquasol and Protosol (New England Nuclear, Boston, MA) were used as scintillation fluid and solubilizer, respectively.

(g) *Membrane filters.*—Centriflo membrane cones (CF50A, Amicon Corp., Lexington, MA) and 0.45 μm Millipore filters (Millipore Corp., Bedford, MA).

Reagents

(a) *Chemicals.*—Neomycin, streptomycin, dihydrostreptomycin, and paromomycin sulfates (U.S. Pharmacopeial Convention, Inc., Rockville, MD); sodium sulfate granular (Mallinckrodt, Inc., St. Louis, MO); 1-pentanesulfonic acid sodium salt, *o*-phthalaldehyde (OPA) (Eastman Kodak Co., Rochester, NY), and other chemicals were reagent grade.

(b) *Solvents.*—Glass-distilled organic solvents (Burdick & Jackson Laboratories, Muskegon, MI) and distilled, deionized water were used throughout the study.

(c) *Post-column derivatization reagent.*—Dissolve 12.36 g (0.2 mol) boric acid in 800 mL water and 56 g (5.0 mol) KOH in 200 mL water. To prepare potassium borate buffer, titrate boric acid solution with KOH solution (30–35 mL) to pH 9.5. Dilute solution with water to 1.0 L. To prepare reagent, dissolve 850 mg OPA in 10 mL methanol, add 2.5 mL 2-mercaptoethanol and 2.5 mL Brij-35, and mix gently until decolorization is complete. Add 1 L potassium borate buffer with mixing. Filter OPA solution through 0.45 μm Millipore filter and refrigerate until used. Subsequently, a commercially available OPA reagent solution (Pierce Chemical Co., Rockford, IL) was used.

(d) *Mobile phase (22).*—0.01M 1-pentanesulfonate–0.056M sodium sulfate–0.007M acetic acid–1.5% (for Supelco and μBondapak/C$_{18}$ columns) or 3% (for Spherisorb ODS-2 col-

umn) methanol, filtered through 0.45 µm Millipore filter and degassed by ultrasonication under vacuum 5 min before use.

(e) *Radiolabeled neomycin.*—Uniformly radiolabeled ^{14}C-neomycin (specific activity 1.08 mCi/mmol) was obtained under FDA contract No. 223-81-7068 with the University of Illinois. A lyophilized sample containing 570 µCi ^{14}C-neomycin was dissolved in 20 mL 2% ethanol for the stock solution. Appropriate dilutions were made as needed.

(f) *Extraction solvent.*—Potassium phosphate buffer (0.2M, pH 8.0) was prepared by dissolving 33.46 g dibasic potassium phosphate and 1.046 g monobasic potassium phosphate in water and diluting to 1 L with water.

(g) *Standard solutions*—Neomycin and paromomycin sulfates were dried 3 h under <5 mm Hg pressure at 60°C. Bottles were capped and placed in desiccator to cool. Stock solutions of 1000 µg/mL of each drug as free base were prepared in polypropylene tubes with water. Aliquots of stock solutions were further diluted with water to give working solutions of 10 µg/mL or as appropriate. All solutions were refrigerated until used. Paromomycin was used as internal standard.

(h) *Ion-pair concentrate.*—A 10-fold solution of ion-pair reagent used in mobile phase was prepared to contain 0.1M 1-pentanesulfonic acid and 0.07M acetic acid, filtered through 0.45 µm Millipore filter, and refrigerated until used.

Standard Curve

All transfers and dilutions were made with Eppendorf digital pipets. From the working solution (10 µg neomycin/mL), 5, 10, 20, 40, 60, and 80 µL aliquots were transferred to separate vials. Aliquots of 100 µL internal standard paromomycin (10 µg/mL) and 20 µL ion-pair concentrate were added to each vial and diluted to 200 µL with water. Vials were vortex-mixed and 50 µL aliquots from each vial were injected onto the LC column.

Extraction and Cleanup Procedures

Extraction.—Frozen, ground control tissue was thinly sliced, and 1 g samples were weighed into tared 50 mL polypropylene tubes fitted with plug-type screw caps. Appropriate aliquots of standard neomycin and paromomycin sulfate solutions were added; tissue-drug mixtures were vortex-mixed and allowed to stand ≥15 min. The samples were homogenized (Tissumizer) 1 min at medium speed in 4.0 mL phosphate buffer; the homogenates were centrifuged 20 min at 3600 × g. The supernate was removed, and the tissue pellet was rehomogenized 10 min as before in the phosphate buffer; supernates were combined in a 15 mL polypropylene tube. Between samples, homogenizer probe was washed with 100 mL water, 10 mL phosphate buffer, and again with 100 mL water; washings were analyzed by LC and no neomycin was detected.

Deproteination.—Polypropylene tubes containing combined tissue extracts were immersed 5 min in boiling water with occasional mixing. Tubes were centrifuged 20 min at 2000 × g, and the deproteinated extract was decanted into another 15 mL polypropylene tube. The precipitated protein pellet was washed with 2 mL phosphate buffer by vortex-mixing about 30 s at high speed, followed by centrifugation 10 min at 2000 × g. The wash supernate was combined with the above deproteinated extract. The combined sample was acidified to pH 3.5–4 with 50–60 µL H_2SO_4, followed by centrifugation for 10 min at 2000 × g. An aliquot of the clear supernate was analyzed directly by LC. The final volumes for kidney and muscle extracts were 9.6 and 7.5 mL, respectively, and the recovery determinations were based on these volumes.

Preparation of Tissue Samples for Recovery of Neomycin

Aliquots of the neomycin sulfate (1000 ppm) stock solution were diluted with water to give concentrations of 600, 300, 200, 100, 50, and 20 ppm. The tissue samples were fortified with 50 µL of each of these solutions to give fortification levels of 30, 15, 10, 5, 2.5, and 1 ppm, respectively. Fifty µL of 1000 ppm paromomycin stock solution was added to a control and to each of the neomycin-spiked tissue samples to serve as an internal standard at a 50 ppm fortification level. The extraction and deproteination of the tissue samples were carried out as described above.

Purification of ^{14}C-Neomycin by LC

[14]C-Neomycin, obtained from the contractor, was purified by LC using a µBondapak/C_{18} column and ion-pairing mobile phase as described above. Initially, the retention time of the reference standard neomycin was established by using post-column derivatization with OPA. This was followed by injection of 100 µL of a 1/10th dilution of the stock solution of ^{14}C-neomycin in the LC column with the Waters U6K injector. Twenty 1 mL fractions were collected. The fractions (usually 3) at the elution position of neomycin were pooled, and an aliquot was counted in the liquid scintillation counter, using 10 mL Aquasol. The pooled fractions contained about 65% of the injected ^{14}C-neomycin and were called "LC-pure" ^{14}C-neomycin.

Use of ^{14}C-Neomycin to Determine Extraction Efficiency

To determine efficiency of extraction of neomycin from kidney tissues, both stock and LC-pure ^{14}C-neomycin solutions were used. Two extracting solvents, saline solution (0.9% NaCl) and pH 8.0 phosphate buffer, were tested to determine their effect on extraction efficiency. Two 1.0 g kidney tissue samples were separately fortified with 7.1 nCi of either stock neomycin or LC-pure neomycin to give fortification levels of ca 4 ppm. The kidney tissue was homogenized in either saline solution or phosphate buffer, and the extraction and deproteination procedures were carried out as described above. Aliquots (1 mL) of the extracts before and after the deproteination step were counted in the liquid scintillation counter, using 10 mL Aquasol, to determine recovery of ^{14}C-neomycin.

Preparation of Extracts and Tissue Residues Containing ^{14}C-Neomycin for Liquid Scintillation Counting

One mL tissue extract was transferred to a 20 mL glass scintillation vial and digested 1 h at 70°C with 4 mL Protosol. The digest was cooled and neutralized with 200 µL acetic acid, mixed with 10 mL Aquasol, followed by warming 1 h at 47°C. The mixture was temperature-equilibrated in the scintillation counter up to 2 h before counting. The extracted kidney residues and precipitated protein pellets were mixed with a small volume of water to yield 1.5 mL homogenate. The homogenates were digested in 6 mL Protosol, and the procedure as described above was followed. The details of the development of this procedure will be reported elsewhere.

Results

LC Separation of Aminoglycosides

Figure 1 shows LC chromatograms of streptomycin, dihydrostreptomycin, and a mixture of paromomycin and neomycin obtained on a Supelcosil LC-8-DB column using the ion-pair mobile phase and post-column derivatization with OPA. Paromomycin and dihydrostreptomycin chromatograms showed the presence of minor impurities, whereas the
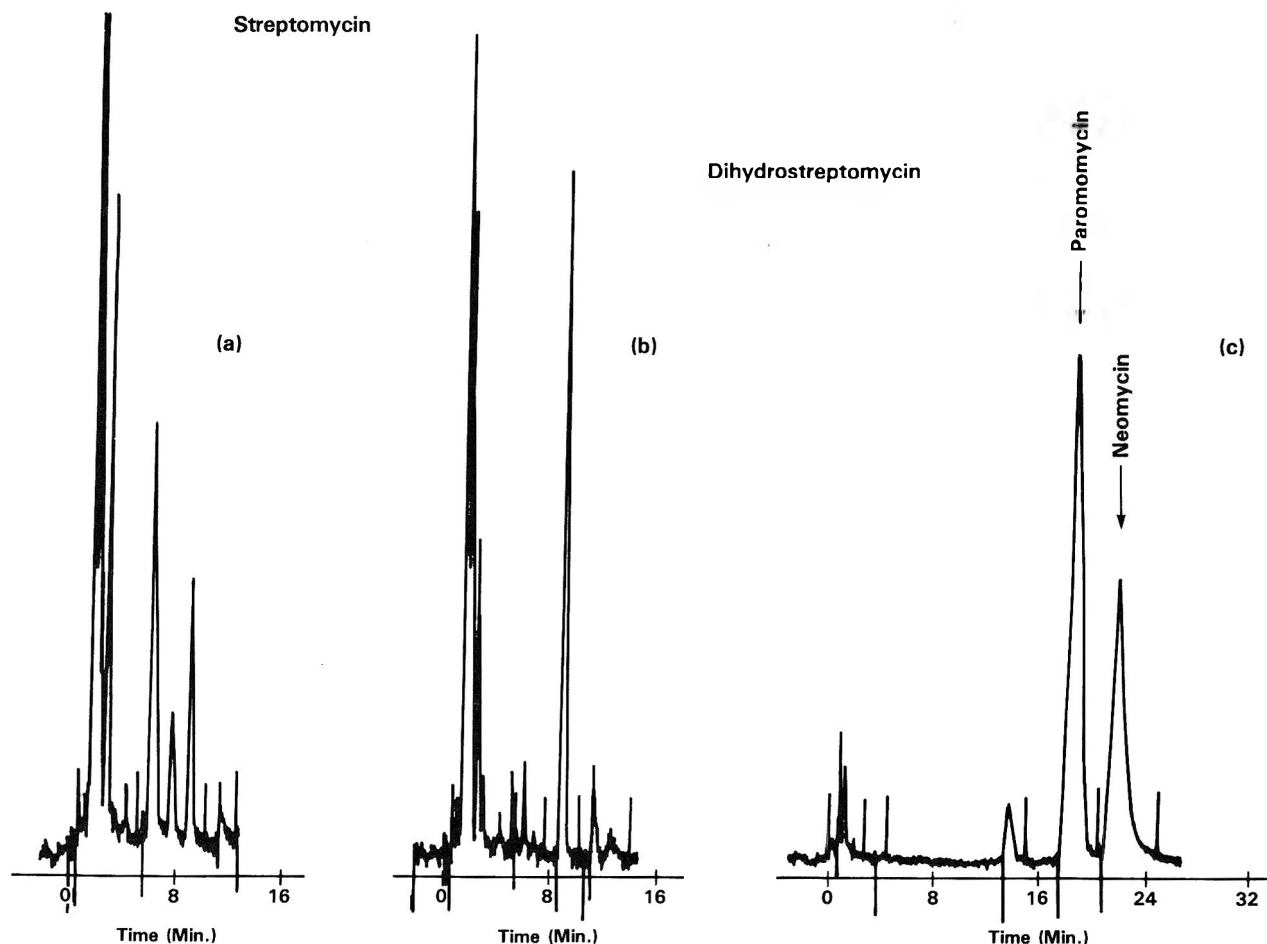
Figure 1.   LC of aminoglycoside standards. a, streptomycin (5 µg); b, dihydrostreptomycin (5 µg); c, paromomycin (250 ng) and neomycin (100 ng). Conditions: column Supelcosil LC-8-DB; mobile phase 0.056M sodium sulfate–0.01M 1–pentanesulfonate–0.007M acetic acid–1.5% methanol; fluorescence detector excitation 340 nm and emission 455 nm; chart speed 0.25 cm/min; flow rate 1.5 mL/min; post-column derivatization with OPA reagent at flow rate of 0.9 mL/min; guard column 10 µm RP-18.

streptomycin chromatogram contained 4 peaks. These peaks represent the parent compound and presumably precursors and/or degradation products of streptomycin (13), the detection of which is enhanced during derivatization with OPA. The first peak, which is the major peak in the chromatogram, most likely represents streptomycin. Neomycin and paromomycin are separable from each other and are retained much longer than dihydrostreptomycin on the LC column; however, dihydrostreptomycin could not be resolved from streptomycin under the conditions used.

Similar retention and separation profiles for these aminoglycosides were obtained on a Supelcosil LC-18-DB column. Other columns examined included µBondapak/C_{18}, Clearsil ODS, and Spherisorb ODS-2. Although all the aminoglycosides were retained on these columns, only Spherisorb ODS-2 resolved neomycin from other aminoglycosides. Streptomycin and dihydrostreptomycin gave similar chromatograms on Spherisorb ODS-2 and Supelcosil LC-8-DB columns. The Spherisorb ODS-2 column also completely separated neomycin from paromomycin and from endogenous compounds in bovine kidney tissue extracts (Figure 2).

The flow rate of OPA that gave optimum sensitivity of detection for neomycin was about ½ the flow rate of the mobile phase. The OPA flow rate of 0.9 mL/min was set on a Kratos post-column reaction system, while the flow rate of the mobile phase was 1.5 mL/min.

Unless indicated otherwise, the Supelcosil LC-8-DB column was used throughout the study. A standard curve in the

range of 5–200 ng neomycin was constructed from the average of duplicate analyses and was linear, with a coefficient of determination (r^2) of 0.998. The average relative retention time of neomycin vs paromomycin was 1.18 ± 0.42% (coefficient of variation, CV, n = 6). The absolute retention times of both drugs, however, were subject to variations in laboratory temperature. The detector sensitivity for the neomycin under the conditions used was about 3.5 ng with a signal-to-noise ratio of about 5.

Recovery of ^{14}C-Neomycin from Kidney Tissues

The results of the recovery of neomycin from kidney tissues fortified with stock and LC-pure ^{14}C-neomycin at the 4 ppm level are shown in Table 1. With saline solutions as the extracting solvent, the recovery of ^{14}C-neomycin was about 60%; this was decreased to 30% after deproteination. The 30% unextracted ^{14}C-neomycin remained in the kidney residue. Table 1 further shows that the recovery of ^{14}C-neomycin was similar when tissue was fortified with either LC-pure (59%) or a stock solution of ^{14}C-neomycin (61%). Therefore, only stock solutions of ^{14}C-neomycin were used in subsequent experiments.

When phosphate buffer was used as the extracting solvent, 90% of the ^{14}C-neomycin was recovered. There were no losses during heat deproteination, resulting in an overall recovery of 90% (Table 1). Therefore, phosphate buffer was selected as the extracting solvent for recovering neomycin from animal tissues in further studies. Initial attempts to deproteinate
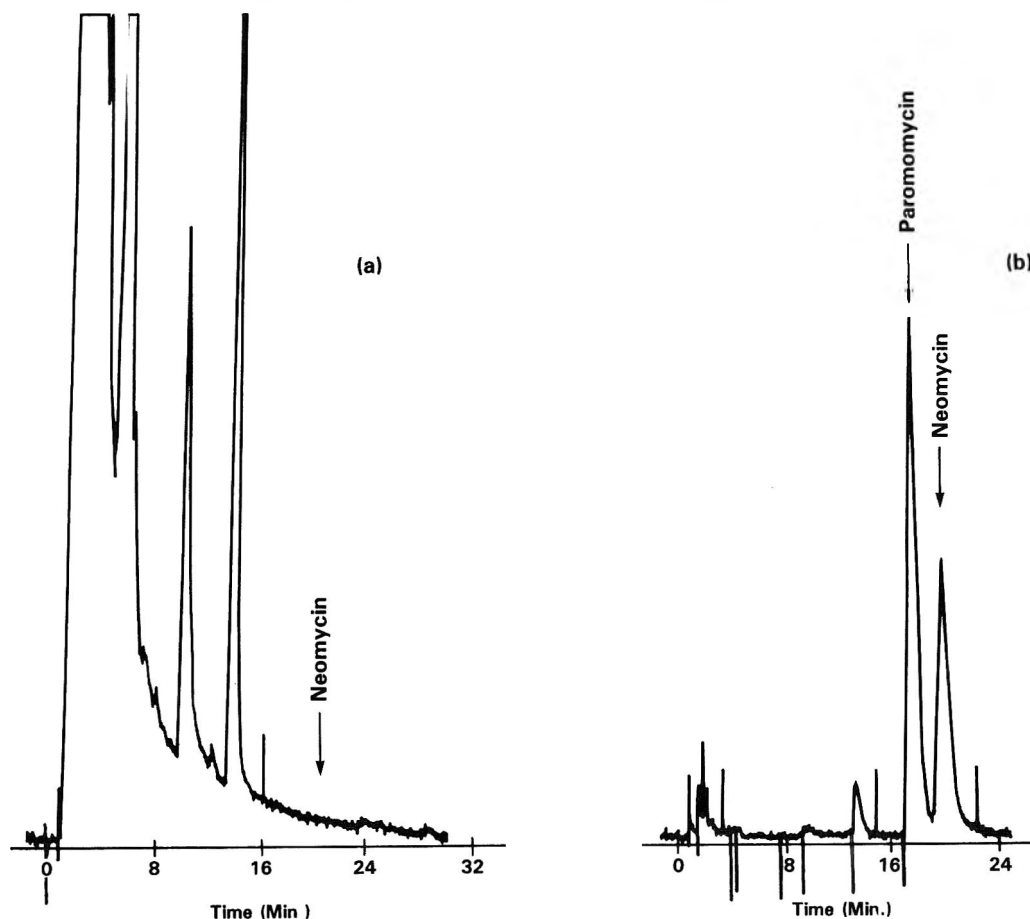
Figure 2. LC of bovine kidney tissue extract and standards on Spherisorb ODS-2 column. a, 25 μL control kidney tissue extract; b, paromomycin (225 ng) and neomycin (90 ng). LC conditions same as Figure 1 except 3% methanol in mobile phase.

Table 1. Recovery of ^{14}C-neomycin[a] from fortified beef kidney tissue

Extract. solv. and fort. level	Rec. from ext, %	Remain. in tissue residue, %	Rec. after deprot. of ext, %	Remain. in protein pellet, %
Saline solution:				
^{14}C-neomycin, LC-pure	62	34	26	—[b]
(7.3 × 10^{-3} μCi, 4.2 ppm)	56	36	20	—[b]
Av.	59	35	23	
^{14}C-neomycin, stock	64	30	29	—[b]
(7.1 × 10^{-3} μCi, 4 ppm)	58	35	28	—[b]
Av.	61	32.5	28.5	
Phosphate buffer (pH 8.0):				
^{14}C-neomycin, stock	97	2	91	4
(7.1 × 10^{-3} μCi, 4 ppm)	84	2	89	5
Av.	90.5	2	90	4.5

[a]Specific activity 1.08 mCi/mmol.
[b]Not analyzed.

the phosphate buffer extract of ^{14}C-neomycin fortified tissues with Centriflo membrane cones, centrifuged 1 h at 1000 rpm, were abandoned because over 50% of the ^{14}C-neomycin was bound to the membrane.

Detection and Quantitation of Neomycin in Fortified Animal Tissues, Using LC

Kidney tissue.—Figure 3 shows chromatograms of 50 μL injections of control and spiked beef kidney tissue samples. Neomycin and paromomycin were well separated from background compounds in the tissue extract.

The recovery of neomycin from fortified kidney tissues at the 1, 2.5, 5, 15, and 30 ppm levels is shown in Table 2. Paromomycin at the 50 ppm level was used as an internal

standard and added to tissue samples along with neomycin at each fortification level. Standards and extracts were injected interspersed to ensure accurate quantitation. The average recovery of paromomycin at the 50 ppm level was 93 ± 9% (*n* = 6). Kidney tissues were also fortified with neomycin without internal standard and carried through extraction, deproteination, and LC analysis; the recoveries at 10 and 30 ppm levels were 90 and 95%, with CVs of 8 and 9% (Table 2). Overall, the recoveries with or without internal standard were similar, i.e., 96 and 93%, with 9.0 and 8.5% CVs, respectively. Use of an internal standard is preferred because it corrects for variations that occur during sample preparation and chromatographic procedures.

The LC profile of control and spiked porcine kidney tissue extracts gave chromatograms (Figure 4) that were similar to those of bovine tissue (Figure 3). The recovery of neomycin at the 10 ppm fortification level was 92 ± 3% (see Table 2).

Muscle tissue.—As for bovine and porcine kidney tissue extracts, the LC profiles of bovine and porcine muscle tissue extracts were similar. Figure 5 shows LC chromatograms of control and spiked bovine muscle tissue extracts. Although neomycin was completely resolved from endogenous compounds in the muscle extract, the background in muscle extracts was higher and different from that seen in kidney extracts. Because paromomycin was not fully resolved from one of the contaminant peaks in muscle extracts, only the neomycin peak was used to determine its recoveries. The recoveries of neomycin at the 10 ppm level from bovine and porcine muscle extracts were 71 and 64%, respectively, with CVs of 4 and 6%.

Table 2. Recovery of neomycin from fortified tissues

Added, ppm	Rec. ± CV, %
Beef Kidney	
1	110
1	92
1	88
Av.	96.7 ± 12.1
2.5	108
2.5	85
2.5	108
Av.	100.3 ± 13
5	79
5	84
5	73
Av.	78.7 ± 6.3
15	92
15	104
15	109
Av.	101.7 ± 8.5
30	100
30	102
30	107
Av.	103.0 ± 3.5
10[a]	83
10[a]	95
10[a]	93
Av.	90.3 ± 7.7
30[a]	92
30[a]	105
30[a]	88
Av.	95.0 ± 9.4
Pork Kidney	
10	96
10	91
10	90
Av.	92.3 ± 3.3
Beef Muscle	
10	74
10	68
10	71
Av.	71.0 ± 4.3
Pork Muscle	
10	67
10	66
10	60
Av.	64.3 ± 5.8

[a]Tissue was fortified only with neomycin; internal standard was added to deproteinated extract immediately before LC analysis.

Specificity of Neomycin Assay

The specificity of the neomycin peak in tissue extracts was established by using peak height ratios obtained from chromatograms run at 2 different emission wavelengths. The peak heights of neomycin in tissue extracts and neomycin standard were determined first at excitation and emission wavelengths of 340 and 455 nm, respectively, and again at 340 (excitation) and 490 nm (emission). The peak height ratio of first vs second chromatograms was calculated for both the neomycin standard and neomycin in the extract. Any deviation from the peak height ratio expected for a particular compound should indicate the presence of another compound eluting near or simultaneously with the compound of interest. In this study, the peak height ratio of neomycin at the above paired wavelength settings for standard (2.30) was almost identical to that of neomycin detected in fortified kidney tissue extract (2.29), indicating that no potential contaminants were co-eluted. Similarly, the peak height ratio for paromomycin standard (2.04) was almost identical to paromomycin detected in tissue extract (2.02). This further indicates that the peak

height ratios of 2 compounds with small structural differences are different. Therefore, in addition to retention time of the peak, using the comparison between peak height ratios of the standard and sample of 2 sets of excitation and emission wavelengths greatly strengthens the validity of the peak identification.

Influence of Guard Column

Both guard columns, Lichrosorb RP-18 and Supelguard LC-8-DB, protected the analytical column during tissue extract analysis. Supelguard, with the same packing material as the analytical column and a shorter length, improved peak symmetry and increased sensitivity, making it possible to detect 1 ppm neomycin-fortified extracts (Figure 6.) Both the guard and analytical columns were flushed with water followed by methanol at the end of the day, and the guard column was replaced after 2–3 months of use. This increased the life of the analytical column to about 6 months.

Use of Ion-Pair Concentrate and pH of Extract

LC chromatograms of both neomycin and paromomycin showed split peaks without addition of the ion-pair concentrate to the sample before injection. Although the problem of split peaks did not occur all the time, it recurred if the ion-pair concentrate was not added to the samples before injection. Therefore, an aliquot of the ion-pair concentrate was added to all the samples before injection to give concentrations of sodium pentanesulfonate and acetic acid equivalent to that in the mobile phase. The wash solvent of the Wisp autosampler was 0.007M acetic acid.

Acidification of the deproteinated extracts to the pH of the mobile phase improved peak shape and also completely resolved the neomycin peak from a later-eluting background peak. Both the acidification of the deproteinated extract and addition of ion-pair concentrate to the sample are vital for consistent chromatography.

Discussion

A rapid, accurate, and sensitive ion-pair reverse phase LC assay for neomycin in tissues has been developed. The method distinguishes neomycin from streptomycin and dihydrostreptomycin. Paromomycin was used as an internal standard because it was adequately separated from neomycin and endogenous background compounds. Using an internal standard increased the reliability and reproducibility of the assay. The method was applied to the detection of neomycin in fortified kidney tissues with recoveries averaging over 90%. Use of ^{14}C-neomycin to fortify tissues facilitated the development of extraction and cleanup procedures. Although both water (8) and saline solution (23) have been used to extract neomycin from tissues, we could recover only 60% of the neomycin with saline solution; however, when phosphate buffer (pH 8.0), which is used in microbiological assays (16) was used, over 90% of the neomycin was recovered from spiked tissues. Heat treatment as a deproteinization step was especially advantageous because essentially all the added neomycin, free and bound, was detected in the tissue extracts, and further dilution by using organic solvents was avoided. Sample extracts and aqueous standards were stored in polypropylene tubes to prevent losses due to adsorption of aminoglycosides on glass containers (22, 24); tissue homogenates were also prepared in polypropylene tubes. Because glass homogenizers lower the recovery of neomycin due to abrasion of glass, which will bind the drug (24), a homogenizer with a metal probe was used.
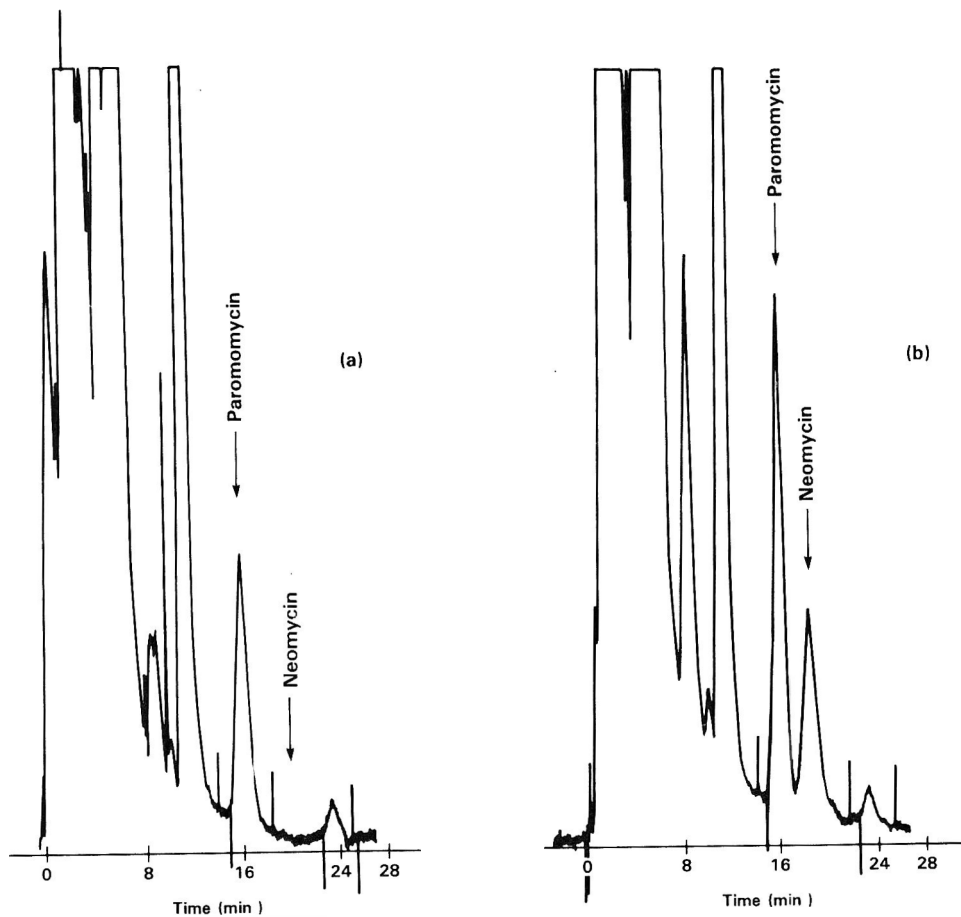
Figure 3.   LC of beef kidney tissue extract on Supelcosil LC-8-DB column. a, 50 µL control kidney tissue extract containing internal standard paromomycin; b, 50 µL kidney tissue extract spiked with paromomycin (250 ng) and neomycin (75 ng). LC conditions same as in Figure 1.
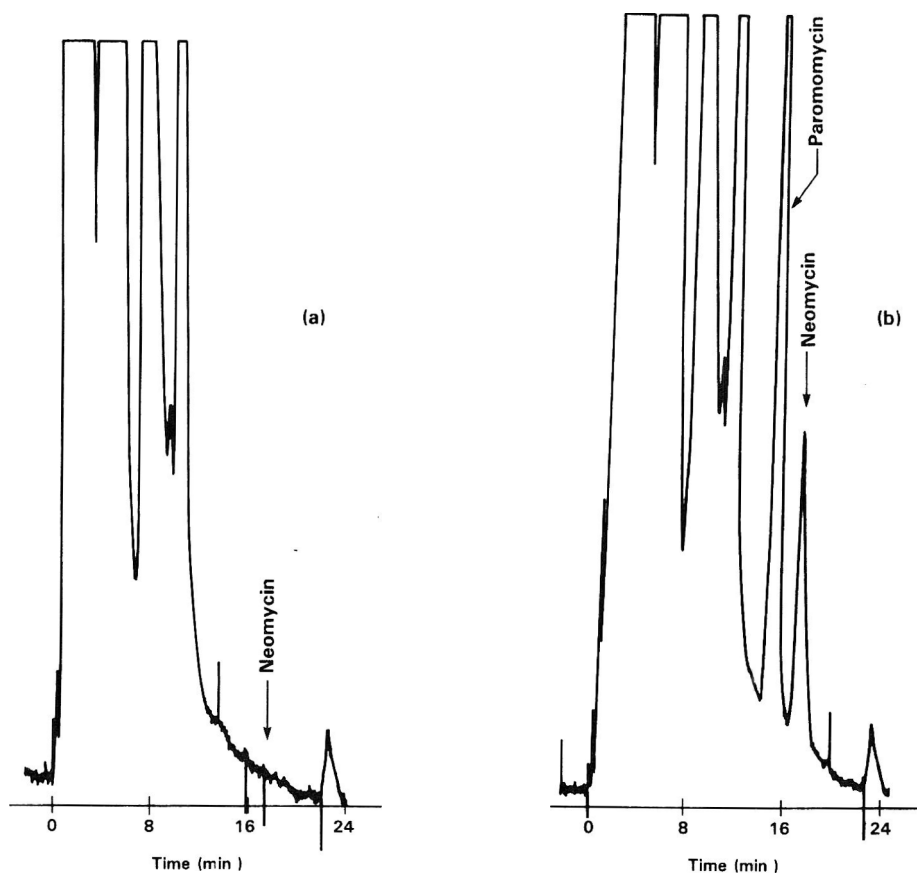
Figure 4.   LC of porcine kidney tissue extracts on Supelcosil LC-8-DB column. a, 25 µL control kidney tissue extract; b, 25 µL kidney tissue extract spiked with internal standard paromomycin (125 ng) and neomycin (25 ng). LC conditions same as Figure 1 except Supelguard used as guard column.
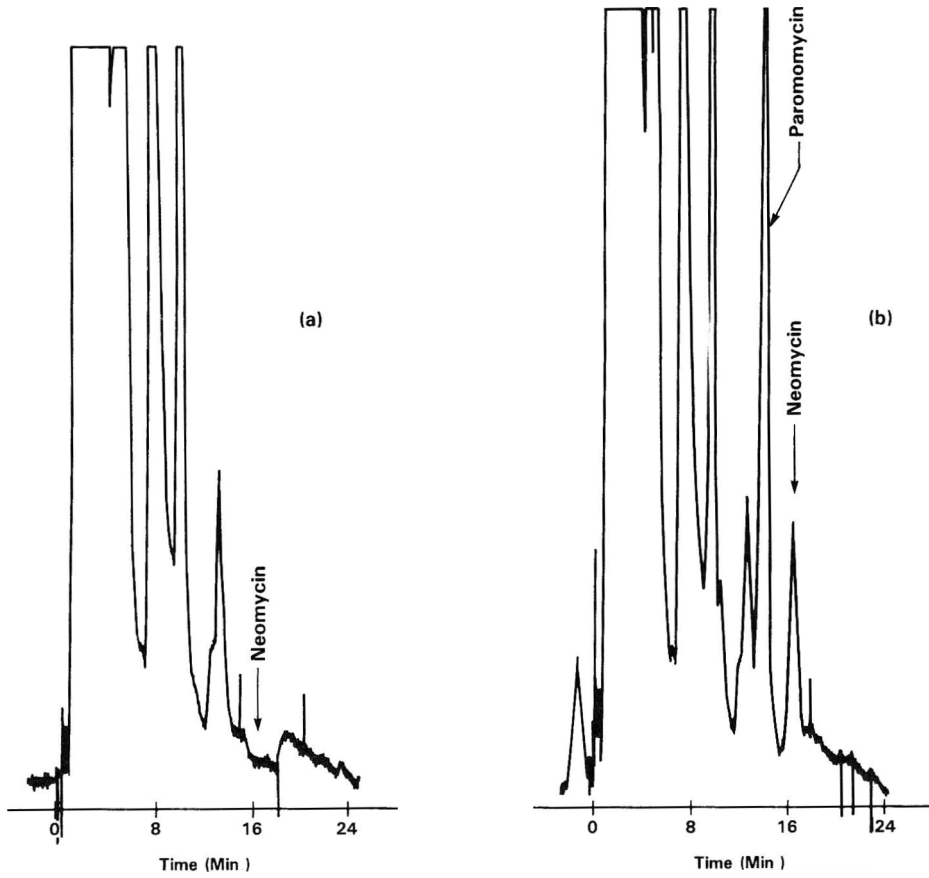
Figure 5.   LC of bovine muscle extracts. a, 25 μL control muscle tissue extract; b, 25 μL muscle tissue extract spiked with internal standard paromomycin (125 ng) and neomycin (25 ng). LC conditions same as Figure 4.
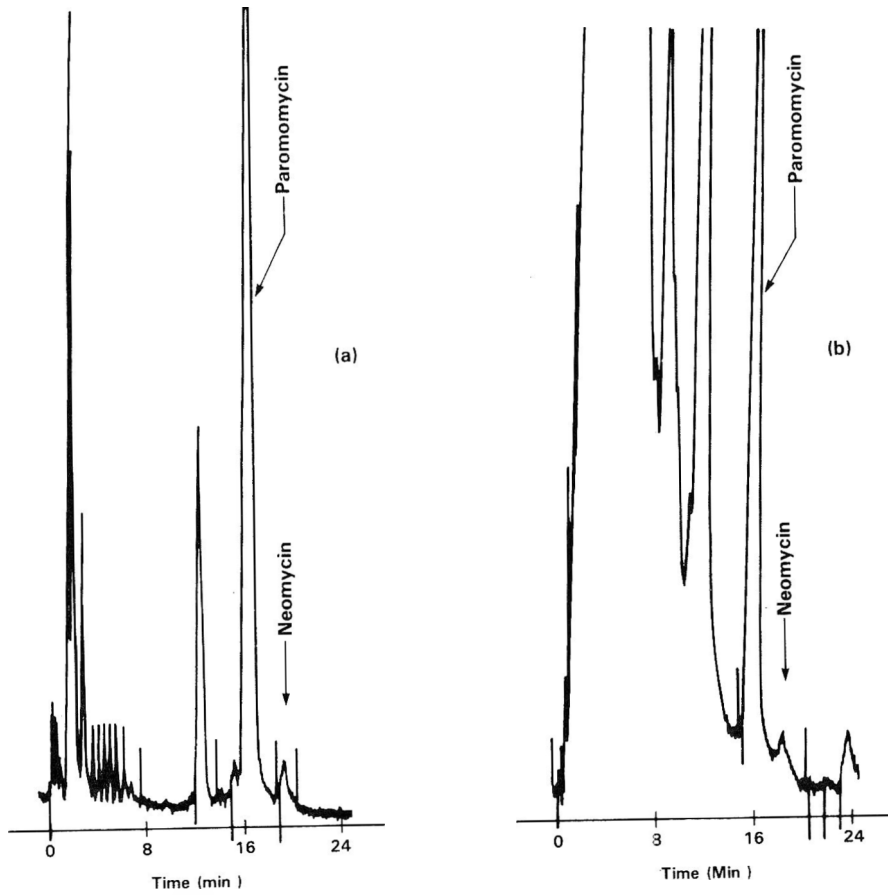
Figure 6.   LC of a, standard, paromomycin (175 ng), and neomycin (3.5 ng); b, 35 μL bovine kidney tissue extract spiked with internal standard paromomycin (175 ng) and neomycin (3.5 ng). LC conditions same as Figure 4.

Various workers (25, 26) have used peak height ratios at 2 or more UV absorption wavelengths to determine the heterogenicity of the peak in question. We used this principle to validate the identity of the neomycin peak in tissue extracts.

With appropriate modifications, this LC technique should be applicable to all aminoglycosides. The major advantage of the present method for determining neomycin over microbiological, enzymatic, or radioimmunoassay methods is that it is specific and has the potential to detect metabolites of the parent drug.

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REFERENCES

(1) Waksman, S. A., Katz, E., & Lechevalier, H. (1950) J. Lab. Clin. Med. **36**, 93–99
(2) Stockley, I. H. (1973) Pharm. J. **210**, 36–43
(3) Waisbren, B. A., & Spink, W. W. (1950) Ann. Intern. Med. **33**, 1099–1119
(4) Nelson, A. A., Radomski, J. L., & Hagen, E. C. (1951) Fed. Proc. **10**, 366–367
(5) Clark, C. H. (1977) Mod. Vet. Pract. **58**, 594–598
(6) Mercer, D. H. (1975) Vet. Clin. North Am. **5**, 3–34
(7) Oehme, F. W. (1973) Toxicology **1**, 205–215
(8) Carli, S., Perretta, G., Monti, F., Sonzogni, O., & Faustini, R. (1982) J. Vet. Pharmacol. Ther. **5**, 203–207
(9) Foppiano, R., & Brown, B. B. (1965) J. Pharm. Sci. **54**, 206–208
(10) Tsuji, K., Robertson, J. H., Baas, R., & McInnis, D. J. (1969) Appl. Microbiol. **18**, 396–398
(11) Decoster, W., Claes, P., & Vanderhaeghe, H. (1982) J. Pharm. Sci. **71**, 987–991
(12) Tsuji, K., Goetz, J. F., VanMeter, N., & Gusciora, K. A. (1979) J. Chromatogr. **175**, 141–152
(13) Whall, T. J. (1981) J. Chromatogr. **219**, 89–100
(14) Inglis, J. M., & Katz, S. E. (1978) Appl. Environ. Microbiol. **35**, 517–520
(15) Katz, S. E., & Levine, P. R. (1978) J. Assoc. Off. Anal. Chem. **61**, 1103–1106
(16) Microbiology Laboratory Guide Book (1974) Scientific Services, Food Safety and Quality Service. U.S. Department of Agriculture, Washington, DC, Part 6, pp. 315–317
(17) Reeves, D. S. (1974) Postgrad. Med. J. **50** (Suppl. 7), 20–21
(18) Nilsson-Ehle, I. (1983) J. Liq. Chromatogr. **6**(Suppl. 2), 251–293
(19) Moats, W. A. (1983) Instrumental Analysis of Foods, Vol. 1, Academic Press, New York, NY, pp. 357–365
(20) Weiss, G., et al. (1983) J. Agric. Food Chem. **31**, 75–78
(21) Bories, G., Peleran, J. C., & Wal, J. M. (1983) J. Assoc. Off. Anal. Chem. **66**, 1521–1526
(22) Anhalt, J. P., & Brown, S. D. (1978) Clin. Chem. **24**, 1940–1947
(23) Daniyelova, L. T. (1973) Antibiotics **15**, 1020–1023
(24) Desrochers, C. S., & Schacht, J. (1981) Experientia **137**, 1357–1358
(25) Krstulovic, A. M., Brown, P. R., & Rosie, D. M. (1977) Anal. Chem. **49**, 2237–2241
(26) Shaikh, B. (1983) J. Chromatogr. **268**, 265–272

Normal Phase Liquid Chromatographic Determination of Nanogram Quantities of Ivermectin in Cattle Blood or Plasma

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A method has been developed for determining ivermectin in 5 mL samples of cattle blood by a 2-step process: cleanup solvent extraction followed by direct injection onto a normal phase liquid chromatography (LC) system with UV detection. Recovery was 77–80% ±5.5% standard deviation. Endogenous interference that may be present caused the lower limit of detection to be set at 4–5 ng/mL. The method was used to show that in blood the distribution of ivermectin favors plasma in a fixed proportion over cellular material, and further to provide a time-course profile of ivermectin in the whole blood of injected cattle. In whole blood, ivermectin concentration peaked between 3 and 5 days and dissipated slowly with a half-life of 3 days.

Ivermectin is a derivative of the biologically active avermectins (1), and is defined as a mixture that contains at least 80% 22,23-dihydroavermectin B_1a (H_2B_1a) and not more than 20% 22,23-dihydroavermectin B_1b (H_2B_1b) (1, 2). Ivermectin is a potent antiparasitic agent (3).

We are interested in control of the cattle tick *Boophilus microplus*, and although ivermectin shows high systemic potency against this pest (4), we consider that future use of this chemical for effective control of cattle ticks will be in sustained, slow-release systems. Because the cattle tick is a bloodsucking pest, a systemically administered chemical will be taken up via the blood meal. As a prerequisite for developing a slow release system, we needed a method for ivermectin analysis sufficiently sensitive to determine systemic levels of therapeutic significance in blood.

Two methods that seemed suitable for use with blood both utilize plasma (5, 6). The first involved extraction, chromatographic cleanup, derivatization, further chromatographic cleanup, and finally injection onto a reverse phase liquid chromatographic (LC) system with fluorometric detection. The method is long and tedious and in our experience was subject to variable and unacceptable losses at the isolation and derivatization steps. The second method, although considerably shorter failed to give satisfactory results, and we were unable to use it on whole blood. This was unacceptable because there was no rational basis for choosing plasma over whole blood as the medium for analysis.

This paper describes a relatively rapid, simple, and accurate method for the determination of ivermectin in whole blood or plasma by extractive cleanup and direct injection onto a normal phase LC system with UV detection. The method was also used to provide data to decide which of plasma or whole blood or either was acceptable as the medium for analysis and to establish a time-course profile for ivermectin in the blood of injected cattle.

Experimental

Standards

H_2B_1a (93% H_2B_1a and 7% H_2B_1b), a gift from Merck, Sharp and Dohme, Australia, was used as the analytical standard for determination of ivermectin. A 0.4 mg/mL solution was prepared in LC grade methanol (3 mL) and was diluted to obtain a 0.004 mg/mL working solution. As required, aliquots were added to silanized tubes and evaporated to dryness at 50°C in a stream of dry air. The linearity of the method was checked in the range 5–200 ng by injecting accurately known
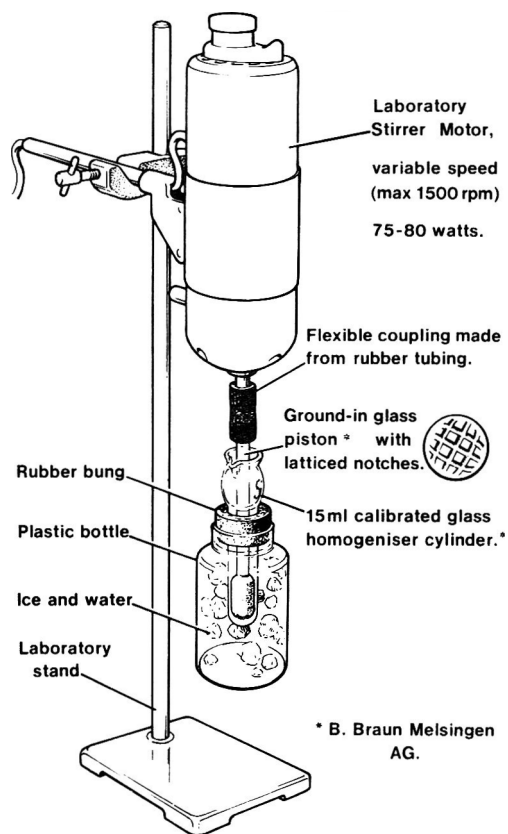
Figure 1.   Homogenizer for extraction of whole blood with ethanol and acetone.

quantities of H_2B_1a directly into the LC system. Recovery and reproducibility were examined by analyzing 6 whole blood and 6 plasma samples spiked with H_2B_1a at 200 ng/5 mL. Recovery was further checked by analyzing whole blood samples spiked with H_2B_1a in the range 5–200 ng/5 mL.

Solvents

n-Hexane, methanol, acetonitrile, ethyl acetate, and tetrahydrofuran (THF) were LC grade solvents. THF was distilled over freshly cut sodium metal to remove water and traces of peroxides. Ethanol and acetone of satisfactory purity were obtained by distilling commerical grade products through a Dufton column. Methylene chloride, dimethylsulfoxide (DMSO), and toluene were AR grade solvents.

Cleaning of Glassware

All glassware was washed thoroughly in hot water and detergent, rinsed in tap water, distilled water, distilled ethanol, and acetone, and silanized as described elsewhere (2), except the silanizing agent was a 2% solution of dimethyldichlorosilane in 1,1,1-trichloroethane.

Liquid Chromatography

A Waters Associates unit equipped with a Model U6K universal injector, 6000A solvent delivery system, 440 detector, and a Cole-Parmer dual pen recorder (0.1–50 mV input) was used with a 30 × 0.39 cm id column packed with Waters Associates μBondapak NH_2 (normal phase adsorbent). Operating conditions: eluting solvent, n-hexane–THF–methanol–DMSO (75 + 25 + 4 + 2), with the latter 3 solvents mixed together before addition to n-hexane, filtered (0.45 μm filter) and degassed before use; flow rate 1 mL/min; ambient temperature (ca 25°C); pressure 800–2000 psi (depending on the number of passes); chart speed 0.5 cm/min; sensitivity 0.01 absorbance unit full scale (AUFS); detector wavelength 254 nm.

Determination

Extraction and cleanup of blood samples.—The method is designed for handling 5 mL whole blood or plasma. Samples can be stored frozen without any apparent deleterious effect at −20°C for ≥6 months before analysis. Samples are extracted and processed as follows:

Add 5 mL ethanol to 5 mL blood in the homogenizer (Figure 1), and homogenize ca 1 min. Centrifuge the homogenizer tubes 5 min at 500× g. Pour supernate into 20 mL centrifuge tube. Repeat operations twice each with 5 mL acetone, combine supernates in 20 mL centrifuge tube, and refrigerate tube 1 h at −20°C. (Samples can be held indefinitely at this stage.)
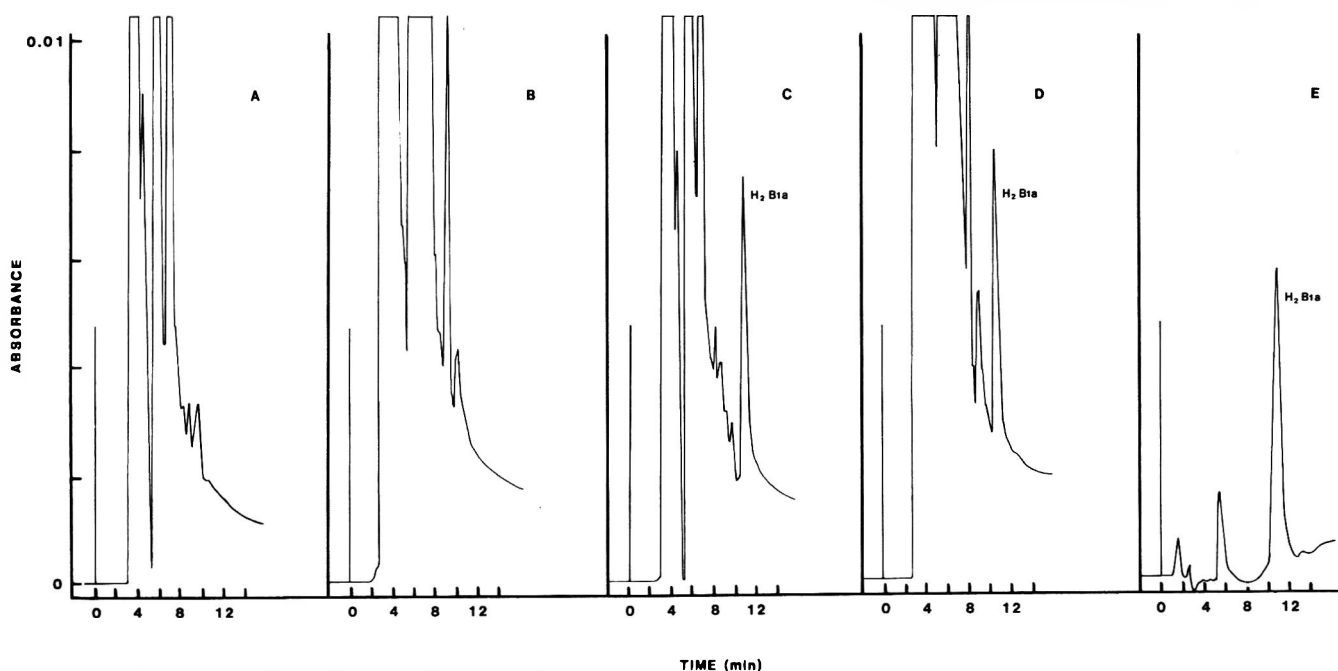
Figure 2.   Typical LC chromatograms of 5 mL blood samples:
A, whole blood blank; B, plasma blank; C, whole blood containing 200 ng 22,23-dihydroavermectin B_1a (H_2B_1a); D, plasma containing 200 ng 22, 23-dihydroavermectin B_1a (H_2B_1a); E, solvent containing 200 ng 22,23-dihydroavermectin B_1a (H_2B_1a). See *Liquid Chromatography* for chromatographic conditions.

Table 1. Recovery of 22,23-dihydroavermectin B₁a from duplicate, spiked, 5 mL whole blood samples

Added, ng	Rec., ng	Rec., %
5	4.9	98
	2.5	50
10	9.8	98
	7.7	77
20	16.9	84
	20.8	104
40	34.3	86
	32.8	82
80	64	80
	61.8	77
160	128	80
	130	81
200	168	84
	174	87

Precipitate that forms remains on reaching ambient temperature. Centrifuge in bench-type centrifuge with swing-out head 5 min at 1000× g. Pour supernate into 25 mL measuring cylinder, and add ethyl acetate equal to ⅕ measured volume. Pour mixture into 15 × 2.5 cm glass-stopper test tube. Add equal volume of glass-distilled water, and mix contents of tube. Extract with 10 mL n-hexane by inverting stoppered tube gently 30 times (n-hexane phase increases in volume). Centrifuge 5 min at 250× g to separate phases. Repeat extraction with another 10 mL n-hexane and combine n-hexane phases in 15 × 1.5 cm glass-stopper test tube. Extract with 4 mL acetonitrile by shaking 30 times. Let phases separate (acetonitrile phase increases in volume) and transfer supernate to 15 mL conical centrifuge tube. Evaporate to near dryness in stream of dry air at 50°C. Repeat extraction with 2 mL acetonitrile and use it to carefully wash down sides of tube containing residue of first acetonitrile extract. Evaporate to dryness. Again, carefully wash down sides of tube with 0.3 mL THF to contain residue within 1 cm from bottom, and evaporate to dryness. Sample is now ready for injection. At this stage, samples may be refrigerated ≥1 week without apparent deterioration. After completing each blood sample analysis, remove tailing extractives by washing column with eluting solvent 4 min at 4 mL/min before injecting next sample.

Injection into LC system.—Inject all standards and sample residues in volume of ca 100 μL as follows: Use 90 μL eluting solvent to wash down residue of standard or sample contained within 1 cm from bottom of 15 mL, conical centrifuge tube. Draw solvent solution into 100 μL syringe. Wash tube with 15 μL eluting solvent (1 cm from bottom) and draw solvent into 100 μL syringe containing first wash. Inject.

Endogenous Interference

During development of the method, we observed that some samples of drug-free blood gave spurious indications of analyte. To determine a statistically acceptable blank value, samples of blood were taken from 6 drug-free steers on 7 separate occasions over a period of 14 days.

Drug Injectables and Sampling

Drug injectables, supplied by Merck, Sharp and Dohme, Australia, contained 10 mg ivermectin/mL. Cattle were injected subcutaneously in the mid-shoulder region at 200 and 500 μg/kg body weight. Blood samples were withdrawn from the jugular vein.

Plasma vs whole blood samples.—No data were available on the distribution of ivermectin between plasma and cellular

material in treated cattle and on the extent to which this distribution varied with time after injection. Three steers (100–150 kg) were injected with 500 μg ivermectin/kg body weight and blood samples were withdrawn on 7 separate occasions over a period of 12 days after dosing. Ten mL aliquots of whole blood were centrifuged 15 min at 1000× g. The plasma was drawn off, and the volume was noted. All of the residue (cellular material), 5 mL of the plasma (the volume recovered from 10 mL of whole blood was rarely < 5 mL), and 5 mL of whole blood were analyzed for ivermectin.

Profile of ivermectin in whole blood.—Three steers were injected with 200 μg ivermectin/kg body weight. Samples were taken at various times over a period of 14 days.

Results and Discussion

The Method

Accuracy and precision.—H₂B₁a elutes with a retention time of 10–13.2 min, depending on the number of passes through the column. It is not resolved from H₂B₁b, the homolog present in ivermectin; however, their structures are similar so the homologs exhibit similar extinctions at 254 nm and peak height accuracy is not affected. Figure 2 shows some elution profiles of blood blanks and samples spiked with H₂B₁a. The extractives load (nonanalyte) in plasma samples is somewhat higher than that in whole blood samples. The linearity of the method over the range 5–200 ng was good; the points conformed closely to a straight line relationship (standard deviation ± 5.4%). Mean recoveries of 200 ng spikes of whole blood and plasma samples were 80% (± 5.5% SD) and 77% (± 5.5% SD), respectively. Table 1 shows recovery of H₂B₁a from whole blood samples spiked in the range 5–200 ng (1–40 ng/mL). No endogenous interference was apparent in the whole blood chosen for spiking. As expected, recoveries varied more at the lower levels. The concentration of analyte in blood was calculated from the formula:

$$ng/mL = 200x/5Ry$$

where R = mean recovery; x = peak height of sample, 0.01 AUFS; and y = peak height of 200 ng standard, 0.01 AUFS.

Taking 1% 0.01 AUFS as the lowest significant peak height, the limit of detection is approximately 3 ng. This represents a blood concentration of 0.6 ng/mL, only 3 times higher than that given for the fluorescence detection method of Tolan et al. (5), the most sensitive method available. That method and its modified form (2), however, require many manipulations and consequently are slow to perform and in our experience prone to losses. We were unable to use the method of Pivnichny et al. (6) on whole blood, which may have been due to our inability to standardize the behavior of the adsorbent Florisil.

Levels and significance of endogenous interference.—Spurious peaks at the ivermectin position were found in both plasma and whole blood samples and in about 50% of the 42 whole blood blank samples analyzed; these provided a mean value of 2.7 ng/mL ± 1.7 SD. The lower limit to which the method is applicable may then become 4–5 ng. A similar problem with endogenous interference was encountered by Tolan et al. (5) and also by Pivnichny et al. (6) for plasma, but the magnitude of the interference is not clear. The interference does not appear restrictive when compared with the peak values associated with the therapeutic doses of 200 and 500 μg/kg body weight (Figure 3 and Table 2, respectively). Furthermore, the emphasis is more on looking at trends from large numbers of samples than on an odd, unusual value.

Table 2. Distribution of ivermectin between plasma and cellular material in blood of cattle injected with 500 μg/kg body weight

Days after injection	Animal	Plasma as fraction of whole blood, v/v	Ivermectin derived from 1 mL whole blood		Ivermectin, ng/mL, in whole blood (W)	Mean and range of fractions, $P/(P + R)$, in plasma	$(P + R) \times 100/W$
			ng in plasma (P)	ng in residue (R)			
1	A	0.62	28	5.4	39	0.82	88
	B	0.48	23	7.0	39	(0.77–0.87)	(77–104)
	C	0.62	65	10	72		
2	A	0.65	31	9.3	55	0.78	93
	B	0.45	35	12	46	(0.77–0.83)	(73–102)
	C	0.62	77	16	91		
5	A	—	—	—	—	0.78	99
	B	0.50	36	12	44	(0.75–0.83)	(89–109)
	C	0.65	55	11	74		
7	A	0.70	40	5.7	38	0.81	114
	B	0.46	26	9.6	33	(0.82–0.87)	(108–120)
	C	0.50	34	7.6	37		
9	A	0.65	32	4.5	36	0.82	101
	B	0.45	22	6.5	28	(0.82–0.88)	(100–101)
	C	0.50	23	5.0	28		
12	A	0.65	15	3.3	15	0.77	123
	B	0.50	11	5.6	14	(0.66–0.82)	(119–124)
	C	0.55	14	3.3	14		
Av.		0.56				0.80	103
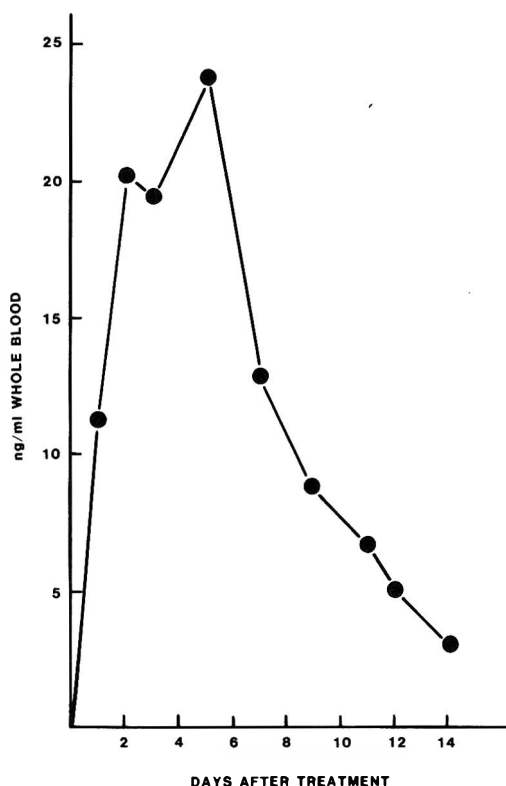
Figure 3. Time-course profile of ivermectin in whole blood of steers injected with 200 μg/kg body weight. Average of blood from 3 steers.

Application of the Method

Plasma vs whole blood.—Table 2 shows the data for these samples. The average plasma volume was 0.56 of the whole blood volume. In all samples, the fraction of ivermectin in the plasma varied little from the average value of 0.8, and the combined fractional values totaled nearly 100%. It appears, therefore, that ivermectin is carried mainly in the noncellular fraction, and its distribution between plasma and blood cells is relatively constant. We conclude that the use of plasma for analysis is quite valid and also advantageous, because ivermectin will be in higher concentration in plasma than in whole blood; however, more manipulations are required because plasma must be separated from whole blood. In most situations, use of whole blood should be satisfactory.

Blood profile.—Figure 3 shows the concentration of ivermectin in whole blood after a single injection. A peak concentration of 24 ng/mL occurred at 5 days, and from that time, ivermectin dissipated slowly. Ivermectin dissipation conformed closely to first-order reaction kinetics. A half-life of 3 days was determined. The inter-animal variation, although high, was randomly distributed over all samplings; the relative variation from the mean was $\pm 37\%$. The reason for this large variation is not known.

General Comments

A freshly prepared column will last for about 150 passes and initially will provide a retention time of 12.2 min for H_2B_1a. With column use, this time increases to a maximum of 13.2 min and then decreases. The peak height also varies slightly with the change in retention. For these reasons, it is necessary to run standards daily with samples. The column should be discarded when the retention time falls to 10 min, usually between 150 and 250 passes.

Occasionally, a sample will record a higher than usual general background. This effect can be nullified by using the offset control to decrease the baseline.

The solvent extraction system was designed specifically for high recovery and cleanup of a sample that is directly injected onto the LC column. The extractives load accompanying the analyte to be injected will be tolerable only by closely following the procedure. The method has been used to determine the ivermectin content of over 700 samples.

After an initial lag of 1 day, 2 operators working in conjunction can process 12 blood samples per day without difficulty.

REFERENCES

(1) Miller, T. W., et al. (1979) *Antimicrob. Agents Chemother.* **15**, 368–371

(2) Tway, P. C., Wood, J. S., & Downing, G. V. (1981). *J. Agric. Food Chem.* **29**, 1059–1063

(3) Campbell, W. C., Fisher, M. H., Stapley, E. O., Albers-Schönberg, G., & Jacob, T. A. (1983) *Science* **221**, 823–828

(4) Nolan, J., Schnitzerling, H. J., & Bird, P. (1981) *Aust. Vet. J.* **57**, 493–497

(5) Tolan, J. W., Eskola, P., Fink, D. W., Mrozik, H., & Zimmerman, L. A. (1980) *J. Chromatogr.* **190**, 367–376

(6) Pivnichny, J. V., Shim, J. K., & Zimmerman, L. A. (1983) *J. Pharm. Sci.* **72**, 1447–1450

FOOD ADDITIVES

Determination of Nitrate in Dried Foods by Gas Chromatography–Thermal Energy Analyzer

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The method described for determining NO_3^- in dried foods is based on extraction of NO_3^- from the sample with subsequent nitration of benzene. The nitrobenzene is extracted with ethyl acetate, analyzed by using a gas chromatograph-thermal energy analyzer (GC-TEA), and quantitated against a nitrobenzene standard. Sensitivity is 100–200 μg/kg. Coefficients of variation for analyses of dried foods were 3–13%. Recovery of NO_3^- from nonfat dry milk spiked at 10 mg/kg averaged 100%.

Recently, there has been increased interest in human exposure to nitrate (NO_3^-) and its relationship to human health (1). Ingestion of NO_3^- may be indirectly associated with certain human cancers (2), infant methemoglobinemia (3), and in vivo nitrosation of N-nitrosamines (4).

White (5, 6) estimates the average dietary intake for a U.S. resident as 100 mg NO_3^- per day, mostly from vegetables and cured meats. Although contributing only minor amounts to the diet, directly drying foods in an open flame increases the NO_3^- content of foods such as nonfat dry milk (7).

Most estimates of the NO_3^- content of foods rely on the reduction of NO_3^- to NO_2^- on a cadmium column, diazotization of sulfanilic acid, and subsequent coupling with N-(1-naphthyl)ethylenediamine to form a chromophore (8). The NO_3^- content is determined as the difference in NO_2^- content of unreduced and reduced samples. This reaction is commonly referred to as the Griess reaction (9) and is the AOAC official method for determining NO_2^- in cured meats (10).

The Griess reaction, with variations, has been used to examine a variety of substances, such as cured meats, cheeses, and vegetables (9), whey powder (11), milk and dried milk products (7), blood (12), and feces (13). A number of interferences (polyphosphates, ascorbate, sulfur dioxide, and low pH) exist for the Griess reaction, which create problems at low levels (10–20 mg/kg) and require tedious cleanup procedures when the method is applied to foods (8). Usher and Telling (8) list other colorimetric methods that are also unreliable because of similar interferences.

Determination of NO_3^- by liquid chromatography (LC) has been developed for aqueous extracts of plant material and tap water (14). Leuenberger et al. (15) have also used LC and 210 nm UV detection to determine NO_3^- in aqueous extracts of vegetables, cheese, and whey. Sensitivity was about 5 mg/kg. Other methods utilizing chemiluminescence apparatus coupled to a thermal energy analyzer (TEA) have been reported for NO_2^-, but these have no adaptations for NO_3^- determination (16), or have limited application to foods because of interference from water (17).

Ross et al. (18) studied the conversion of NO_3^- to nitrobenzene with measurement by gas chromatography–electron capture detection and reported the method suitable for water and air samples. They further extended application to environmental samples (19).

Wu and Saschenbrecker (20) have also used the nitration of benzene in a method for simultaneously estimating NO_2^- and NO_3^- in meat products. Our laboratory simplified the nitration procedure and applied it to the estimation of NO_3^- in blood, urine, saliva, and feces (21). The present work applies the nitrobenzene procedures to determination of NO_3^- in dried food products.

METHOD

Reagents

(a) *$ZnSO_4$ and Ag_2SO_4.*—Prepare aqueous solution containing both 0.48M $ZnSO_4$ (Mallinckrodt, Inc., Paris, KY 40361, No. 8880) and 7.7 mM Ag_2SO_4 (Fisher Scientific Co., Fair Lawn NJ 07410, No. S-190).

(b) *$Ba(OH)_2$.*—Prepare aqueous saturated solution (Mallinckrodt, Inc., No. 3772).

(c) *Benzene.*—Aldrich Chemical Co., Milwaukee, WI 53233, No. 15630-2.

(d) *H_2SO_4.*—Concentrated (Mallinckrodt, Inc., No. 2468).

(e) *NaOH.*—Prepare 5N aqueous solution (Mallinckrodt, Inc., No. 7708).

(f) *Ethyl acetate.*—Mallinckrodt, Inc., No. 4992.

(g) *Nitrobenzene.*—Dilute standard serially with ethyl acetate to 5.0 μg/mL or within the sample range (Aldrich Chemical Co., No. N1-095-0).

Apparatus

(a) *Centrifuge.*—Damon/International Equipment Co. high-speed, refrigerated centrifuge, Model B-20A, with 50 mL fixed angle rotor No. 870 with polycarbonate tubes.

(b) *Filter.*—Kimax filter funnel with fused-in fritted disc, 60 mL coarse frit.

(c) *Gas chromatograph (GC)–thermal energy analyzer (TEA).*—Aerograph Model 200 chromatograph interfaced to Thermo Electron analyzer Model 543. Instrumental conditions: 3.05 m × 4 mm stainless steel column packed with 3% OV-17 or 3% OV-25 on 100–120 mesh Chromosorb WHP; helium carrier gas 25 mL/min; temperatures (°C): injector 190, column 140, TEA interface 175, pyrolyzer 875; pressure 2.2 torr; cold trap −175°C (liquid nitrogen cooling).

(d) *Glassware.*—Thoroughly clean all glassware, including a chromic acid rinse, before each analysis.

Determination

Prepare 1:5 (w/v) aqueous solution of nonfat milk powder, nondairy creamer, whey powder, or buttermilk powder, or 1:10 dilution (w/v) of instant coffee, cocoa powder, or chewing tobacco. Filter chewing tobacco suspension.

Pipet. 2.0 mL sample into 50 mL polycarbonate centrifuge tube and heat 2 min in boiling water bath. Add 1.0 mL $ZnSO_4$/Ag_2SO_4 solution and 0.5 mL $Ba(OH)_2$, in that order, to samples in bath. Continue heating 5 min. Centrifuge 10 min at 15 krpm and filter supernate, if necessary, with fritted glass filter. To test tube on ice, add 0.5 mL supernate, 25 μL benzene, and 1.0 mL H_2SO_4. Heat 15 min at 70°C, mixing frequently. Cool tubes in ice. Add 2.0 mL 5N NaOH while vortex-mixing and cool on ice. Add 0.5 mL ethyl acetate,

vortex-mix, and let layers separate. Make duplicate 6–8 μL injections of ethyl acetate layer (upper layer) into GC-TEA apparatus, and quantitate peaks with proper retention times against 8 μL injections of external nitrobenzene standard. Calculate NO_3^- concentration as follows:

$$4.41 \; P \; (C) \; V'/P' \; V = \mu g \; NO_3^-/g \; sample \; (dry \; wt), \; or$$

$$8.82 \; P \; (C) \; V'/P' \; V = \mu g \; NO_3^-/g \; sample \; (dry \; wt)$$

where 4.41 = factor for conversion of nitrobenzene to NO_3^- for 1:5 dilution; 8.82 = factor for conversion of nitrobenzene to NO_3^- for 1:10 dilution; P = peak height of sample; P' = peak height of nitrobenzene standard; C = concentration (μg/mL) of nitrobenzene standard; V = injection volume (μL) of sample; and V'' = injection volume (μL) of nitrobenzene standard. All injections are made at the same attenuation. Concurrently analyze and subtract reagent blanks from unknowns.

Table 1. Analysis of several different products for nitrate

Product	N	X, μg NO_3^-/g	SD, μg NO_3^-/g	CV, %
Nonfat milk	14	12	1.1	9
Instant coffee 1	6	96	7.6	8
Instant coffee 2	4	18	0.7	4
Instant coffee 3	4	46	1.8	4
Nondairy creamer 1	6	13	0.9	7
Nondairy creamer 2	4	37	1.9	5
Nondairy creamer 3	4	11	0.3	3
Buttermilk powder	3	8.9	0.35	4
Baby formula	4	9.8	0.98	10
Dried whey powder	4	4.3	0.58	13
Cocoa powder	4	49	2.9	6
Chewing tobacco	4	13 000	1400	10

Results and Discussion

The sensitivity of this method is 100 μg/kg for NO_3^- in nonfat dry milk, nondairy creamers, baby formulas, and dried whey powder. All other products had a sensitivity of 200 μg/kg because of the higher original dilution. This sensitivity is similar to that of the method of Tesch et al. (19) for analysis of environmental and biological samples with benzene nitration; about 10 times lower than that of the method reported for dried products, using the Griess reaction (9, 11); and those of LC procedures (15). Under the operating conditions described above, our GC–TEA instrument gave a 3-to-1 signal-to-noise ratio (×2) when 0.1–0.2 ng nitrobenzene (which represents about 0.05–0.1 ng NO_3^-) was injected. Linearity was demonstrated ($r^2 > 0.99$) by analyzing samples spiked at 0.17–1435 mg/kg.

Recoveries of NO_3^- added to a sample of nonfat dry milk (10 mg/kg) were 111, 125, 109, 113, 93, 108, 86, 67, 93, 89, 109, and 99%, with an average recovery (n = 12) of 100% (s = 16%, range = 67 – 125%). The relatively large standard deviation in recovery is likely due to the presence of 10 mg NO_3^-/kg in the sample before spiking. Unspiked samples were analyzed concurrently with spiked samples. We were unsuccessful in locating a nonfat dry milk sample with less than 10 mg/kg of NO_3^-. These recovery data compare favorably with those reported in nonfat dry milk (7) spiked at 1.25 mg/kg and analyzed by a diazotization coupling reaction. Our recoveries are similar to those reported by Sen and Lee (11), who spiked dried whey at 30–50 mg/kg and corrected recoveries for the efficiency of their cadmium column reduction.

We applied our technique to a number of other dry products to investigate the general applicability to this class of products (Table 1). The concentration of NO_3^- in food products gen-
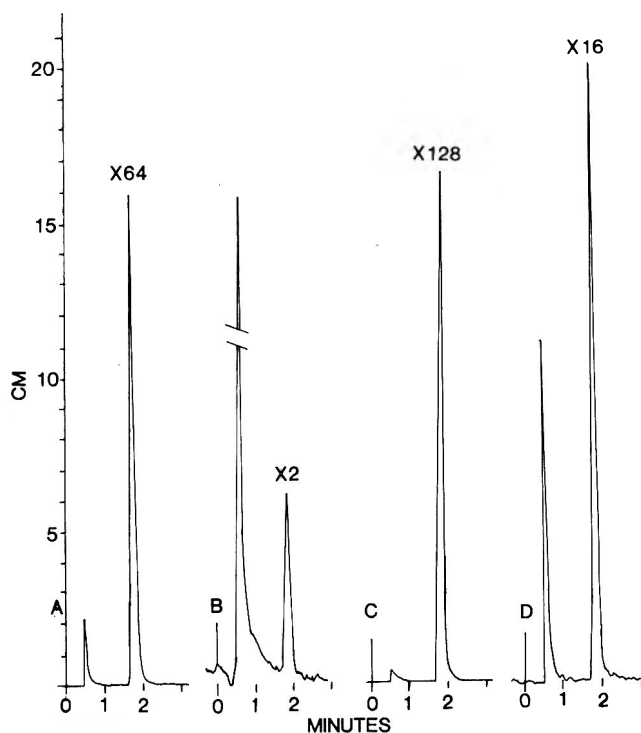
Figure 1. GC-TEA chromatograms of A, 27.7 ng nitrobenzene standard; B, reagent blank; C, extract of instant coffee containing 48.7 mg/kg of NO_3^-; D, extract of nonfat dry milk containing 6.4 mg/kg of NO_3^-.

erally ranged from 5 to 100 mg/kg. Chewing tobacco (snuff) contained 13 000 mg/kg. Coefficients of variation for repeated analyses ranged from 3 to 13%. With the exception of the tobacco product, all products are diluted with water before consumption and the levels of NO_3^- represent a small fraction of daily NO_3^- ingestion.

As noted in the AOAC official method (10), filter papers, if used, should be thoroughly washed. In our work, Whatman filters were occasionally a major source of NO_3^- contamination. For this reason, we routinely centrifuged and/or used fritted glass filters in place of paper.

The thermal energy analyzer is both a sensitive and selective detector for nitroaromatic compounds. All products that we analyzed produced simple chromatograms, containing one peak (Figure 1). All chromatograms in Figure 1 came from an OV-25 column. Retention times on OV-17 were 10–12% longer. The peak height, and hence the sensitivity, is related to the temperature of the pyrolyzer tube in the TEA furnace (Figure 2). No response was seen below 700°C, and the response continued to increase to 925°C, the highest temperature tested. At any given temperature, response varied less than ± 3% of peak height. Electron capture detection would likely produce sensitivity similar to that of TEA, as would N-P type detectors. Flame ionization detection can be used with a subsequent decrease in sensitivity and specificity. Neither pigmentation nor the precipitate that occurred in some samples interfered with the analyses.

While we do not report NO_2^- in these products, this procedure can be used to estimate NO_2^- if aqueous extracts are treated with $KMnO_4$ (20) or H_2O_2 (19) to oxidize the NO_2^- to NO_3^- before nitration. The NO_2^- concentration is then estimated by substrating an unoxidized NO_3^- aliquot from an oxidized one. We found that 0.01 mL 30% H_2O_2 added to the 2.0 mL aliquot was sufficient to effect quantitative oxidation.

Our procedure has advantages of speed, simplicity of sample preparation, and specificity. Quantities of sample are small and blanks normally exhibit low background NO_3^-;
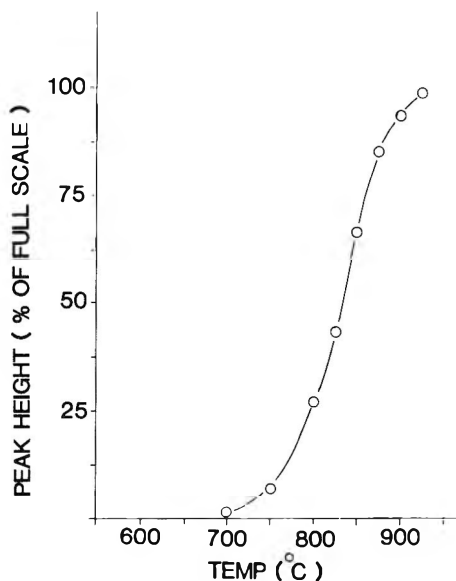
Figure 2. Effect of TEA pyrolyzer temperature on detector response (peak height) for 12.3 ng nitrobenzene injected (attenuation ×64; full scale = 27 cm).

H_2SO_4 is the major source of background due to contamination with NO_3^-. Care must be exercised, however, because NO_3^- is widely dispersed and contamination is a potential problem. The ethyl acetate layer may be separated from the aqueous layer and stored ($-5°C$). The major disadvantage is the necessity of using specialized detectors (TEA or EC) for maximum sensitivity.

Acknowledgment

REFERENCES

(1) National Academy of Sciences (1981) *The Health Effects of Nitrate, Nitrite, and N-Nitroso Compounds*, Part 1, The National Academy Press, Washington, DC
(2) Hartman, P. E. (1983) *Environ. Mut.* **5**, 111–112
(3) Archer, M. C. (1982) *Nutritional Toxicol.* **1**, 328–381
(4) Ohshima, H., & Bartsch, H. (1981) *Can. Res.* **41**, 3658–3662
(5) White, J. W. (1975) *J. Agric. Food Chem.* **23**, 886–891
(6) White, J. W. (1976) *J. Agric. Food Chem.* (correction) **24**, 202
(7) Manning, P. B., Coulter, S. T., & Jenness, R. (1968) *J. Dairy Sci.* **51**, 1725–1730
(8) Usher, C. D., & Telling, G. M. (1975) *J. Sci. Food Agric.* **2**, 1793–1805
(9) Sen, N. P., & Donaldson, B. (1978) *J. Assoc. Off. Anal. Chem.* **61**, 1389–1394
(10) *Official Methods of Analysis* (1984) 14th Ed., AOAC, Arlington, VA, secs **24.044–24.045**
(11) Sen, N. P., & Lee, Y. C. (1979) *J. Agric. Food Chem.* **27**, 1277–1279
(12) Schneider, N. R., & Veary, R. A. (1973) *Am. J. Vet. Res.* **34**, 133–135
(13) Archer, M. C., Saul, R. L., Lee, L. J., & Bruce, W. R. (1981) *Banbury Report No. 7, Gastrointestinal Cancer: Endogenous Factors*, P. N. Magee (Ed.), Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, p. 321
(14) Thayer, J. R., & Huffaker, R. C. (1980) *Anal. Biochem.* **102**, 110–119
(15) Leuenberger, U., Gauch, R., Reider, K., & Baumgartner, E. (1980) *J. Chromatogr.* **202**, 461–468
(16) Doerr, R. C., Fox, J. B., Lakritz, L., & Fiddler, W. (1981) *Anal. Chem.* **53**, 381–384
(17) Walters, C. L., Downes, M. J., Hart, R. J., Perse, S., & Smith, P. L. R. (1978) *Z. Lebensm. Unters. Forsch.* **167**, 229–232
(18) Ross, W. D., Buttler, G. W., Duffy, T. G., Rehg, W. R., & Wininger, M. T. (1975) *J. Chromatogr.* **112**, 719–727
(19) Tesch, J. W., Rehg, W. R., & Sievers, R. E. (1976) *J. Chromatogr.* **126**, 743–755
(20) Wu, W., & Saschenbrecker, P. W. (1977) *J. Assoc. Off. Anal. Chem.* **60**, 1137–1141
(21) Dull, B. J., & Hotchkiss, J. H. (1984) *J. Food Chem. Toxicol.* **22**, 105–108

VETERINARY ANALYTICAL TOXICOLOGY

Atomic Absorption Spectrophotometric Determination of Liver Copper: Collaborative Study

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Eleven collaborating laboratories conducted replicate analyses on 4 blind duplicate pairs of bovine liver samples that either had naturally acquired copper levels or were spiked with one of 3 copper levels. A National Bureau of Standards Bovine Liver sample (SRM 1577, 193 ± 10 mg copper/kg) and a 1000 mg copper/L standard were also submitted to the collaborators. The method requires the tissue to be digested with concentrated HNO₃ at 60°C, diluted to volume with water, and analyzed by atomic absorption spectrophotometry. The intralaboratory coefficients of variation (CV_o) ranged from 5.6 to 19%; the interlaboratory CV_x values ranged from 7.1 to 21%. The lower limit of detection was estimated to be 1 mg copper/kg tissue. The method has been adopted official first action.

Copper is an essential element in several enzyme systems and in biochemical reactions that influence or control such diverse body functions as pigmentation of hair and wool, utilization of iron in the production of hemoglobin, fertility, and cardiovascular and neuro-motor activities (1, 2). Animals deficient in copper may be unthrifty and suffer a wide range of health problems (1–3).

Copper toxicity can occur in ruminants, notably sheep, that consume more copper than required. This may lead to copper accumulation in the liver, occurrence of a hemolytic crisis evident as an ecterus, and eventual death from damage to liver and kidney tissues (4).

The determination of copper in animal serum for the diagnosis of copper deficiency or toxicosis has been reported previously (5). While serum may be the only sample available for the diagnosis of a nonfatal copper toxicosis or deficiency, liver and kidney are the diagnostic samples of choice if the animal dies or is sacrificed.

Collaborative Study

The proposed method, which employs a wet-ashing technique reported by Braselton et al. (6), was submitted to 12 collaborating laboratories along with 4 blind duplicate pairs of bovine liver samples, a National Bureau of Standards Bovine Liver sample (SRM 1577, 193 ± 10 mg Cu/kg), and a 1000 mg Cu/L standard. The laboratories were required to assay each sample in replicate on different days.

Samples for the collaborative study were prepared as follows: The tissue was cut into 1 cm pieces, which were dropped individually into liquid nitrogen (LN₂) and then ground with additional LN₂ in a Stein mill. The ground liver, while still frozen, had the consistency of finely divided powder. After thawing, the liver was blended in a Waring blender, and aliquots were then removed for spiking and further blending. Samples sent to collaborating laboratories consisted of 4 blind

duplicate pairs, all prepared from the same batch of ground bovine liver as follows:

Sample Pairs	Intended Spike
1, 2	Liver
3, 4	Liver + 10 mg Cu/kg
5, 6	Liver + 25 mg Cu/kg
7, 8	Liver + 280 mg Cu/kg

Copper in Liver
Atomic Absorption Spectroscopic Method
First Action

Principle

One g liver tissue is digested overnight at 60° in 5 mL HNO₃, then dild to 25 mL with H₂O and analyzed by AAS.

Apparatus and Reagents

(a) *Atomic absorption spectrophotometer (AAS).*—Equipped with nebulizer and 10 cm, air–C₂H₂ burner head. Monitor performance by assuring that 4.0 mg Cu/L std produces ≥0.200 absorbance unit.

(b) *External control.*—Standard Reference Material (SRM 1577) Bovine Liver (193 ± 10 mg Cu/kg, U.S. Dept of Commerce, National Bureau of Standards, Washington, DC 20243) or equiv.

(c) *Nitric acid.*—Concd and dild (1 + 4).

(d) *Teflon screw-cap bottles.*—30 mL wide mouth (Cole-Parmer, K-6103-30) or equiv.

(e) *Copper std solns.*—*(1) Stock std soln.*—1000 mg Cu/L. Dissolve 1.000 g Cu metal in 10 mL HNO₃–H₂O (1 + 1). Dil. to 1000 mL with 1% HNO₃. *(2) Intermediate std soln.*—100 mg Cu/L. Dil. 10 mL stock std soln to 100 mL with H₂O. *(3) Working std solns.*—Dil. 0.0, 0.25, 0.5, 1.0, 2.0, and 4.0 mL intermediate std soln to 100 mL with HNO₃ (1 + 4) to give Cu stds contg 0.0, 0.25, 0.5, 1.0, 2.0, and 4.0 mg Cu/L, resp.

Sample Preparation

Rinse all glassware with 2N HCl. Mix samples thoroughly before weighing. Into sep. Teflon screw-cap bottles, accurately weigh 1.0 g liver tissue and 0.25 g external control, recording both wts to nearest 0.01 g. Use one external control for each 10 samples or fraction thereof. (Note: Complete digestion will not occur for >0.5 g dry wt of samples or controls.) Add 5 mL concd HNO₃ to each bottle, tighten cap, and place bottles overnight in 60°, ventilated oven.

Remove bottles from oven and cool to room temp. Using H₂O to rinse bottles, transfer sample digests to 25 mL vol. flasks, allowing any fat to remain adhering to digestion bottles. Dil. flasks to vol. with H₂O.

Determination

Analyze by AAS using following conditions: wavelength 324.7 nm; slit 0.7 nm; flame air–C₂H₂ (lean-blue). Aspirate series of working std solns, external control solns, and sample dilns. Prep. std curve of concn (mg Cu/L) vs A and det. sample

Table 1. Collaborative data[a,b] for AAS determination of copper in bovine liver tissue

Lab.	Samples[c]							
	1	2	3	4	5	6	7	8
1	1.7	3.0	16	16	32	32	290	300
	3.2	4.0	18	17	33	33	280	290
2	4.3	4.6	17	15	29	27	290	260
	3.6	3.9	16	15	26	27	270	260
3	4.2	3.8	16	16	30	31	290	290
	3.9	3.7	17	17	30	31	280	280
4	2.9	2.9	18	14	33	31	305[d]	390[d]
	5.0	5.4	19	19	38	32	300[d]	340[d]
5	3.6	4.1	17	17	32	32	300	290
	3.9	3.8	17	16	33	31	300	280
6	3.8	3.8	16[d]	16[d]	32	32	260	270
	4.6	4.6	14[d]	14[d]	26	27	260	270
7	4.2	4.6	18	18	30	32	300	300
	4.2	4.1	17	16	31	31	290	290
8	3.9	3.9	17	16	30	33	290	270
	4.3	6.9	18	18	34	33	310	300
9	3.9	3.9	17	17	31	31	270	270
	3.1	3.1	16	16	30	30	290	300
10	3.8	4.7	16	16	30	30	300	290
	3.6	3.7	17	16	30	31	280	290
11	3.3	3.3	18	17	34	34	290	290
	2.8	3.8	16	16	36	30	290	270

[a]Units are mg Cu/kg tissue.
[b]Results of replicate analyses of blind duplicate sample pairs.
[c]For spiking levels, see Collaborative Study.
[d]Outliers according to the Dixon test, but were included in statistical analyses.

Table 2. Statistical data from collaborative study[a]

	Samples			
	1,2	3,4	5,6	7,8
Mean (mg/kg)	3.9	17	31	290
Repeatability:[b]				
S_o	0.74	1.1	1.7	16.2
CV_o (%)	19	6.5	5.6	5.6
Reproducibility:[c]				
S_x	0.8	1.2	2.4	22
CV_x (%)	21	7.1	7.8	7.6
Recovery, %	—	—[d]	108	102

[a]The lower limit of detection is 1 mg Cu/kg liver tissue.
[b]Includes intralaboratory and day-to-day variation.
[c]Interlaboratory variation.
[d]Error in 10 mg Cu/kg spike preparation; no calculation of recovery was made.

by determining that a 0.04 mg Cu/L standard had absorbance readings >300% above background.

The method can be equally effective when used with other biological tissue such as kidney.

Recommendation

The Associate Referee recommends that this method be adopted official first action.

soln concns. Dil. with HNO_3 (1 + 4) any samples above range of working stds. Repeat analysis if external control Cu value is not within accepted range. Calc. mg Cu/kg tissue (X):

$$X = (C \times 25 \times D)/W$$

where C = sample soln concn (mg Cu/L); D = additional sample diln; and W = tissue wt (g).

Results and Discussion

Results were received from 11 collaborating laboratories (Table 1). One laboratory used peak heights on a strip chart recorder rather than absorbance units for quantitation. One laboratory experienced leakage of fumes from the Teflon bottles during digestion. This problem was solved by pairing the bottles and caps. Because Teflon flows, a cap and bottle may conform to one another over time and should remain paired to ensure the tightest seal. One collaborator suggested use of less expensive polyethylene bottles instead of Teflon. Two laboratories reported problems with fat particles clogging the AAS aspirator tube. Allowing the digests to cool to room temperature before transferring them to the volumetric flasks ensures that fat will adhere to the sides of and remain in the digestion vessel.

An error was made in the preparation of the 10 mg Cu/kg spike; therefore, no calculation of recovery was made for this level. The mean recoveries of the 25 and 280 mg Cu/kg spiked pairs were 108 and 102%, respectively. Statistical analysis was performed according to the AOAC Statistical Manual (7). Although averged results from Laboratory 4 on samples 7 and 8 and Laboratory 6 on samples 3 and 4 were outliers according to the Dixon test, all results returned from the collaborators were used to calculate the statistical values reported in Table 2. Previous research indicates that collaborative studies on results given in parts per million produce interlaboratory precision values of about ±15% (8, 9). Interlaboratory precision values in this study were within that range and support the validity of this procedure for diagnostic use. The lower limit of detection was estimated to be 1 mg Cu/kg tissue

REFERENCES

(1) Piscator, M. (1979) in Handbook on the Toxicology of Metals, L. Friberg & G. F. Nordberg (Eds), Elsevier/North-Holland Biomedical Press, New York, NY, pp. 411–420

(2) Underwood, E. J. (1977) Trace Elements in Human and Animal Nutrition, 4th Ed., Academic Press, New York, NY, pp. 56–108

(3) Buck, W. B., & Osweiler, G. D. (1976) in Clinical and Diagnostic Veterinary Toxicology, G. Van Gelder (Ed.), Kendall/Hunt, Dubuque, IA, pp. 297–314

(4) Van Ryssen, J. B. J. (1982) Proceedings of the Georgia Nutrition Conference for Feed Manufacturers, Atlanta, GA, pp. 89–97

(5) Osheim, D. L. (1983) J. Assoc. Off. Anal. Chem. 66, 1140–1142

(6) Braselton, W. E., Meerdink, G. L., Stowe, H. D., & Tonsager, S. R. (1981) Proceedings of the 24th Meeting of the American Association of Veterinary Laboratory Diagnosticians, St. Louis, MO, pp. 111–125

(7) Youden, W. J., & Steiner, E. H. (1975) Statistical Manual of the AOAC, AOAC, Arlington, VA

(8) Horwitz, W. (1983) J. Assoc. Off. Anal. Chem. 66, 1295–1301

(9) Mason, M. F. (1981) J. Anal. Toxicol. 5, 201, 208

OILS AND FATS

Gas Chromatographic Determination of *Trans* Unsaturation in Margarine: Collaborative Study

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An international collaborative study of a gas chromatographic (GC) method for the determination of *trans* unsaturation in margarine was conducted in 1980. Thirteen collaborators analyzed a set of 2 of 3 known mixtures of methyl esters and 4 margarine methyl ester samples. Two of the margarine methyl ester samples were blind duplicates. The experimental data were subjected to statistical analysis to determine within- and between-laboratory variation. The statistical data were in excellent agreement with data from a collaborative study of the AOAC-IUPAC GC method for the determination of methyl esters of fatty acids. Coefficients of variation (CVs) for components in the range of 4–40% were comparable (CV 2–6% for soybean oil methyl esters (AOAC-IUPAC study) as well as for margarine methyl esters (this study)). Good recoveries of total *trans* acids were obtained and between-laboratory as well as within-laboratory CVs were consistently better for the determination of total *trans* acids by GC vs infrared analysis. The GC method for determining *trans* unsaturation in margarine has been adopted official first action.

An international collaborative study was carried out in 1980 of a packed column gas chromatographic (GC) method (1) for determination of *trans* unsaturation in methyl esters of fatty acids in margarine. The method requires a 20 ft (6.1 m) × 2 mm id glass or stainless steel column packed with 15% OV-275 coated on 100–120 mesh Chromosorb P AW-DMCS for GC analysis of the fatty acid methyl esters, using a flame ionization detector. The method may also be useful for estimating fatty acid composition along with simultaneous determination of *trans* content, although the OV-275 column is less suitable than other columns for determining fatty acid composition.

Collaborative Study

A set of 2 of 3 standard methyl ester mixtures and 4 samples of methyl esters of commercially available margarines (total of 6 samples) were sent to 17 collaborators. Two of the margarine methyl esters (Samples ct-4 and ct-6)[1] were blind duplicates.

Each collaborator was provided with instructions to follow the procedure exactly as described by Perkins et al. (1). It was also requested that columns be purged with carrier gas 2–3 h before raising the column temperature for conditioning and each time the column was exposed to air. The collaborators were requested to analyze each sample once, except analyze Sample ct-4 in duplicate and report as ct-4-A and ct-4-B. The *trans* content of each sample determined by the infrared (IR) method (2) was requested for comparison to the total *trans* value (18:1t + 18:2ct + tt) obtained by the GC method. The collaborators were asked to submit the peak area percentage of each component in the samples together with a typical chromatogram and all GC conditions used.

Total *Trans* Fatty Acid Isomers in Margarines
Gas Chromatographic Method
First Action

(Not applicable to samples contg hydrogenated marine oils)

Principle

Me esters of fatty acids from margarines are sepd and measured by GC to det. total *trans* unsatn content (*trans* isomers of unsatd 18 C acids). Results by this method are comparable to those obtained by IR method **28.086–28.091**.

Apparatus

(a) *Gas chromatograph.*—With flame ionization detector and min. dead space in injection system. Maintain column temp. within ±1° at ca 220°.

(b) *Column.*—6.1 m (20 ft) × 2 mm id glass or stainless steel (polyunsatd components with > 3 double bonds may decompose in stainless steel column).

(c) *Packing.*—Acid-washed and silanized diat. earth, 100–120 mesh, coated with 15% OV-275 stationary phase (15% OV-275 on 100–120 mesh Chromosorb P AW-DMCS is available from Supelco). OV-275 columns are extremely sensitive to O, particularly at operating temp. Carrier gas and all lines must be free of O. Condition new columns by purging with carrier gas overnight at room temp. and then gradually increasing temp. (1–2°/min) to temp. 10–20° higher than operating temp. but no higher than recommended limits. If column has been removed from instrument or exposed to air, purge column with carrier gas for 1–2 h before heating.

(d) *Syringe.*—Max. vol. 10 μL, graduated to 0.1 μL.

(e) *Recorder.*—0–2.5 or 5.0 mV range, <1.0 s response rate (time for pen to pass from 0 to 90% following momentary introduction of 100% signal), 25 cm/min paper width, and 25–100 cm/h paper speed; equipped with attenuator switch to change range. If integrator is used, it must have linear response with adequate sensitivity and satisfactory baseline correction.

Reagents

(a) *Carrier gas.*—He (preferred for better resolution and column life) or N dried and contg ≤ 10 mg O/kg.

(b) *Other gases.*—H, 99.9⁺% free from org. impurities. Air or O, free from org. impurities (<2 ppm hydrocarbons equiv. to CH_4).

(c) *Reference stds.*—Mixt. of *cis* and *trans* Me esters of known composition close to that of margarines.

Preparation of Sample

Isolate fat by **16.236**. Using ca 350 mg fat. prep. Me ester by **28.056–28.059**. To Me esters in g-s flask, add vol. of heptane to yield 1–10% soln. Swirl to dissolve.
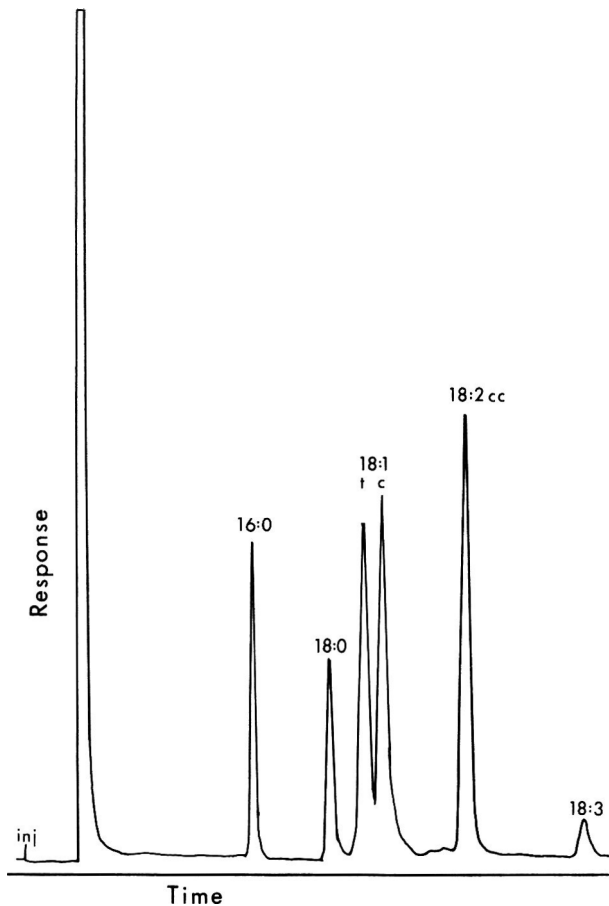
Fig. 28:A1. Typical chromatographic separation of methyl esters of fatty acids from margarines. c = *cis*; t = *trans*. Not shown are components 18:2tt, 18:2ct + tt, and 20:0, which elute between 18:1c and 18:2cc.

GC Operating Conditions

Carrier gas flow depends on carrier gas and packing density. Following flow rates are recommended: He, 15–20 mL/min; N, 8–10 mL/min. Flow of H to detector should be half that of carrier gas; flow of O should be ca 5–10 times that of H. Injector should be at 20–50° higher than column temp. and detector should be at 250–300°.

GC Performance Specifications

Perform analysis of mixt. of Me elaidate and Me oleate. Adjust sample size, column temp., and carrier gas flow so that Me elaidate peak is recorded ca 30 min after injection and ½–¾ full scale. Measure base widths in mm of Me elaidate (w_1) and Me oleate (w_2) between points of intersection with baseline of tangents drawn to inflection points of curves. Also measure distance in mm between peak max. for elaidate and Me oleate, Y. Calc. resolution, R:

$$R = 2Y/(w_1 + w_2)$$

Select conditions to obtain $R \geq 0.5$.

Determination

With app. showing stable baseline, inject 0.1–2 μL 1–10% heptane soln of Me esters. Change setting of attenuator as necessary to keep peaks on chart paper.

Analyze ref. std mixts under same conditions as for sample. Measure retention distances of known esters and identify peaks from sample by comparison with retention distances of peaks from std mixts. Fig. **28:A1** shows a typical chromatographic separation of methyl esters of margarine fatty acids.

Table 1. Operating conditions reported by collaborating laboratories[a]

Lab.	Carrier gas/flow rate, mL/min	Column temp., °C	Column
1	helium, 8	220	stainless steel
2	nitrogen, 10	220	stainless steel
3	helium, 30	220	stainless steel
4	helium, 5.4	220	stainless steel
5	helium, 17	225	stainless steel
6	nitrogen, 9	220	glass
7	—[b]	220	—[b]
8	nitrogen, 8	245	stainless steel
9	nitrogen, 9	205	—[b]
10	helium, 12	220	stainless steel
11	helium, 10	220	stainless steel
12	nitrogen, 9	220	glass
13	helium, 20	220	stainless steel

[a] All laboratories used a 20 ft (6.1m) × 2 mm id column packed with 15% OV-275 stationary phase as specified in the method.
[b] Not reported.

Calculations

Use method of internal stdzn (normalization), which assumes all components of sample are represented on chromatogram, so that sum of areas under peaks represents 100% of constituents (total elution). Obtain total *trans* content by addn of all *trans* isomers.

Ref.: *J. Am. Oil. Chem. Soc.* **54**, 279(1977).

Results and Discussion

Analytical results were received from 13 of the 17 laboratories to which samples were sent. Nine collaborators reported using stainless steel columns; 2 collaborators reported using glass columns. Use of either a glass or stainless steel column did not affect the results. Seven collaborators reported using helium carrier gas; 5 collaborators used nitrogen carrier gas. Carrier gas flow rates ranged from 5 to 30 mL/min (Table 1). Again, there was no indication that use of either nitrogen or helium carrier gas or a particular flow rate affected the results.

The experimental data received from the collaborators (Tables 2–9) were subjected to statistical analysis to obtain descriptive statistics associated with analysis of each of the samples as well as to obtain within- and between-laboratory variability from results of analysis of a blind duplicate sample. Tables 10–12 show results of statistical analysis of the collaborative data. No outliers were omitted; only 4 of 158 total *trans* values were Dixon outliers (2 values each for total *trans* by GC and total *trans* by IR). Total *trans* determined with IR averaged about 1% higher than total *trans* determined by GC, apparently resulting from IR addition of each *trans* bond in *trans, trans* components and/or GC coelution of minor *trans* components with major *cis* acids. Although Perkins et al. (1) have reported good separation of 18:2ct + tt from 18:2cc in margarine methyl esters with a 20 ft OV-275 column, one collaborator observed that 18:2tt was not separated from 18:1c in the samples. This collaborator also noted that 18:2cc was not resolved from 20:0. Another collaborator reported that 18:2ct and 18:2tt were not resolved from 18:2cc. Although not shown in Fig. **28:A1**, components 18:2tt, 18:2ct + tt, and 20.0 elute between peaks 18:1c and 18:2cc

Two collaborators also analyzed the samples with Silar-10C capillary columns, and obtained results similar to those with the OV-275 packed column. One of the collaborators noted that, although the capillary peaks were difficult to identify at first, with some experience capillary column analysis was preferred, one advantage being its speed. With margarine samples, about 30 components were separated on capillary

Table 2. GC analysis of margarine methly esters (Sample ct-3)

Lab.	16:0	18:0	18:1t	18:1c	18:2 ct + tt	18:2 cc	18:3	Total trans GC	Total trans IR
				% of total fatty acids[a]				Total trans	
1	11.5	8.4	15.1	33.3	6.6	22.0	1.3	21.7	23.0
2	11.2	8.3	15.0	34.0	6.7	23.2	1.0	21.7	23.8
3	13.3	8.3	15.3	34.1	6.4	22.7	—[b]	21.7	24.4
4	11.6	8.2	15.2	36.0	5.8	21.9	0.9	21.0	22.5
5	11.4	8.4	15.0	34.2	6.4	22.3	1.1	21.4	24.4
6	11.1	8.3	15.1	31.4	6.9	22.8	1.1	22.0	24.0
7	11.5	8.4	15.2	34.6	6.8	22.5	0.8	22.0	22.1
8	12.6	8.3	14.2	34.7	6.6	22.1	1.7	20.8	—[c]
9	11.2	8.1	14.0	33.5	6.3	25.0	1.2	20.3	24.0
10	11.3	8.5	15.1	32.8	7.7	22.9	1.7	22.8	20.8
11	12.3	8.4	15.4	35.3	6.0	21.4	0.8	21.4	22.8
12	11.3	8.4	15.5	34.7	6.4	21.7	0.9	21.9	23.9
13	11.0	8.3	15.3	32.8	7.4	22.7	1.7	22.7	—[c]
x̄, %	11.6	8.3	15.0	34.0	6.6	22.6	1.1	21.6	23.2
SD	0.58	0.10	0.44	1.20	0.51	0.90	0.47	0.70	1.1
CV, %	5.8	1.2	2.9	3.6	7.8	4.0	42.7	3.2	4.8

[a]t = trans; c = cis; ct + tt = cis, trans + trans, trans; cc = cis, cis.
[b]No value reported; 0.0 assumed.
[c]No IR analysis.

Table 3. GC analysis of margarine methyl esters (Sample ct-4-A)

Lab.	16:0	18:0	18:1t	18:1c	18:2 ct + tt	18:2 cc	18:3	Total trans GC	Total trans IR
1	10.5	9.1	21.7	25.0	0.9	27.9	3.5	22.6	22.2
2	10.9	9.0	21.5	25.8	0.5	28.9	3.3	22.0	23.3
3	11.1	9.2	22.2	25.7	—[b]	28.7	3.2	22.2	23.9
4	11.4	8.8	21.7	26.1	0.4	28.0	3.3	22.1	22.5
5	10.8	9.2	21.6	25.5	0.8	27.9	3.5	22.4	23.5
6	10.6	9.0	21.6	25.0	1.0	28.4	3.3	22.6	22.0
7	10.5	9.8	22.3	26.3	0.1	28.1	3.0	22.4	22.8
8	11.9	9.4	21.7	28.3	0.1	25.7	3.0	21.8	—[c]
9	10.9	8.9	21.0	26.5	1.0	28.5	3.2	22.0	22.4
10	11.3	9.2	21.9	25.3	1.4	27.2	3.7	23.3	20.4
11	11.8	9.4	21.8	27.6	—[b]	26.8	2.0	21.8	24.2
12	10.5	9.0	21.7	26.5	0.2	28.2	3.3	21.9	24.7
13	10.5	9.1	21.8	24.4	1.4	28.6	3.7	23.2	—[c]
x̄, %	11.0	9.2	21.7	26.0	0.6	27.9	3.2	22.3	22.9
SD	0.50	0.26	0.32	1.08	0.51	0.89	0.43	0.49	1.20
CV, %	4.5	2.8	1.5	4.1	85.5	3.2	13.3	2.2	5.2

[a]See Table 2, footnote a.
[b]No value reported; 0.0 assumed.
[c]No IR analysis.

Table 4. GC analysis of margarine methyl esters (Sample ct-4-B)

Lab.	16:0	18:0	18:1t	18:1c	18:2 ct + tt	18:2 cc	18:3	Total trans GC	Total trans IR[b]
1	12.4	9.0	21.8	25.3	0.8	26.9	3.2	22.6	—
2	11.0	9.0	21.5	25.8	0.5	28.9	3.3	22.0	—
3	10.7	9.3	22.2	25.8	—[c]	28.8	3.2	22.2	—
4	11.0	8.9	21.7	26.2	0.4	28.1	3.3	22.1	—
5	10.8	9.1	21.6	25.6	0.8	27.9	3.4	22.4	—
6	10.6	9.0	21.6	25.0	1.0	28.4	3.3	22.6	—
7	10.9	9.7	22.3	26.1	0.1	28.0	3.0	22.4	—
8	11.8	9.5	22.1	28.3	0.1	25.2	3.0	22.2	—
9	10.8	8.8	20.6	26.5	1.1	28.9	3.2	21.7	—
10	11.2	9.6	21.9	25.0	1.6	26.6	4.1	23.5	—
11	11.6	9.4	22.0	27.0	—[c]	27.2	2.2	22.0	—
12	11.0	9.1	21.5	25.7	0.3	28.2	3.3	21.8	—
13	10.4	9.1	21.8	24.8	1.3	28.6	3.8	23.1[d]	—
x̄, %	11.1	9.2	21.7	25.9	0.6	27.8	3.3	22.4	—
SD	0.55	0.28	0.43	0.95	0.53	1.08	0.44	0.51	—
CV, %	4.9	3.1	2.0	3.7	85.7	3.9	13.4	2.3	—

[a]See Table 2, footnote a.
[b]No IR analysis.
[c]No value reported; 0.0 assumed.
[d]Dixon outlier at 95% confidence level.

Table 5. GC analysis of margarine methyl esters (Sample ct-5)

Lab.	16:0	18:0	18:1t	18:1c	18:2 ct + tt	18:2 cc	18:3	GC	IR
				% of total fatty acids[a]				Total *trans*	
1	10.7	5.5	5.6	31.9	3.3	37.2	3.4	8.9	9.4
2	11.0	5.5	5.6	32.8	2.8	33.7	3.0	8.4	11.1
3	11.0	5.7	5.8	32.0	3.1	39.0	2.9	8.9	11.6[b]
4	10.6	5.6	5.7	35.3	2.8	37.1	2.6	8.5	9.4
5	10.6	5.5	5.4	32.5	3.3	37.8	3.2	8.7	9.2
6	10.5	5.7	6.0	31.9	3.5	37.8	3.0	9.5	10.0
7	10.7	5.7	5.8	33.4	3.5	33.2	2.7	9.3	9.5
8	11.7	5.8	4.9	34.6	3.2	37.2	2.5	8.1	—[c]
9	10.8	5.5	5.2	32.6	3.4	38.9	2.7	8.6	9.5
10	10.8	5.2	5.9	32.1	4.1	37.7	4.0	10.0	8.4
11	10.8	5.7	5.6	33.7	3.3	36.5	2.7	8.9	9.6
12	10.5	5.7	6.1	33.3	3.4	36.9	2.8	9.5	9.3
13	10.2	5.6	5.9	31.3	4.1	37.4	3.7	10.0	—[c]
$\bar{x}$, %	10.8	5.6	5.7	32.9	3.4	37.7	3.0	9.0	9.7
SD	0.35	0.16	0.33	1.15	0.39	0.79	0.45	0.60	0.90
CV, %	3.3	2.8	5.8	3.5	11.7	2.1	14.9	6.7	9.3

[a] See Table 2, footnote a.
[b] Dixon outlier at 95% confidence level.
[c] No IR analysis.

Table 6. GC analysis of margarine methyl esters (Sample ct-6)

Lab.	16:0	18:0	18:1t	18:1c	18:2 ct + tt	18:2 cc	18:3	GC	IR
				% of total fatty acids[a]				Total *trans*	
1	10.8	9.1	21.6	24.9	0.8	27.8	3.4	22.4	21.6
2	10.5	9.2	21.7	26.1	0.9	28.5	3.2	22.6	24.1
3	11.0	9.2	22.3	25.8	—[b]	28.6	3.2	22.3	23.8
4	10.9	8.8	21.8	26.4	0.4	28.0	3.3	21.7	22.1
5	10.8	9.1	21.7	25.5	0.8	27.9	3.4	22.5	23.5
6	10.5	9.1	21.6	25.0	1.0	28.3	3.4	22.6	21.0
7	10.1	9.5	22.6	26.3	0.1	28.3	3.0	22.7	21.9
8	12.2	9.4	21.5	28.4	0.1	25.6	2.9	21.6	—[c]
9	11.2	9.2	21.2	26.9	0.6	27.8	3.1	21.8	23.5
10	10.9	9.2	21.4	25.1	1.8	28.0	3.6	23.2	20.8
11	11.0	9.7	21.9	27.2	—[b]	27.7	2.3	21.9	21.9
12	10.9	9.1	21.9	25.1	0.4	28.4	3.2	22.3	24.3
13	10.5	9.1	21.7	24.8	1.4	28.2	3.9	23.1	—[c]
$\bar{x}$, %	10.9	9.2	21.7	26.0	0.6	27.9	3.2	22.4	22.6
SD	0.49	0.22	0.39	1.07	0.55	0.76	0.38	0.49	1.3
CV, %	4.5	2.4	1.8	4.1	86.9	2.7	11.8	2.2	5.8

[a] See Table 2, footnote a.
[b] No value reported; 0.0 assumed.
[c] No IR analysis.

Table 7. GC analysis of methyl ester standard No. 3

Lab.	16:0	18:0	18:1t	18:1c	18:2 ct + tt	18:2 cc	18:3	GC	IR
				% of total fatty acids[a]				Total *trans*	
1	10.8	10.3	5.7	30.7	1.5	37.2	3.6	7.2	7.8
3	11.0	10.2	6.9	30.9	1.1	36.3	3.5	8.0	9.5[b]
4	11.1	9.8	5.7	30.6	2.0	37.2	3.6	7.7	6.8
5	10.9	10.5	5.7	31.5	1.2	36.1	3.3	6.9	7.2
6	10.7	10.3	5.8	30.5	2.3	36.6	3.5	8.1	8.0
7	10.7	10.3	5.7	31.6	2.3	36.5	3.1	8.0	7.7
9	11.5	10.0	5.5	30.4	1.4	37.6	3.6	6.9	7.1
10	11.4	10.5	5.8	30.8	0.6	37.3	3.7	6.4	6.9
11	11.0	10.6	5.8	33.6	0.9	35.3	2.8	6.7	4.8[b]
13	10.4	10.2	5.8	30.0	1.5	36.9	3.9	7.3	—[c]
$\bar{x}$, %	11.0	10.3	5.8	31.1	1.5	36.7	3.5	7.3	7.3
SD	0.33	0.24	0.38	1.01	0.57	0.69	0.32	0.60	1.25
CV, %	3.0	2.3	6.6	3.3	38.7	1.9	9.2	8.2	17.1
Known value, %	10.3	10.0	5.6	30.0	1.1	38.3	3.9	6.7	6.7
Rec., %	106.8	97.1	103.6	103.7	136.4	95.8	89.7	109.0	109.0

[a] See Table 2, footnote a.
[b] Dixon outlier at 95% confidence level.
[c] No IR analysis.

Table 8. GC analysis of methyl ester standard No. 4

Lab.	% of total fatty acids[a] 16:0	18:0	18:1t	18:1c	18:2 ct+tt	18:2 cc	18:3	Total trans GC	IR
1	10.6	5.1	15.7	25.5	2.5	37.6	2.9	18.2	19.2
2	10.6	5.1	15.4	25.9	2.5	37.8	2.7	17.9	20.7
3	10.9	5.1	15.7	26.0	2.2	37.4	2.7	17.9	21.5
4	11.0	5.0	15.4	25.3	3.2	37.3	2.8	18.6	18.4
6	10.5	5.2	15.5	25.4	3.3	37.2	2.8	18.8	20.0
9	10.7	5.2	15.2	26.0	2.3	37.8	2.8	17.5	16.4
12	10.9	5.3	16.1	25.9	1.8	37.3	2.7	17.9	19.3
13	10.4	5.2	15.4	25.1	2.5	37.4	2.9	17.9	—[b]
$\bar{x}$, %	10.7	5.2	15.6	25.6	2.5	37.5	2.8	18.1	19.4
SD	0.21	0.09	0.28	0.35	0.50	0.23	0.08	0.43	1.66
CV, %	2.C	1.8	1.8	1.4	19.6	0.6	3.0	2.4	8.6
Known value, %	10.2	5.1	15.5	25.0	2.1	38.3	3.1	17.6	17.6
Rec., %	104.9	102.0	100.6	102.4	119.0	97.9	90.3	102.9	110.2

[a]See Table 2, footnote a.
[b]No IR analysis.

Table 9. GC analysis of methyl ester standard No. 5

Lab.	% of total fatty acids[a] 16:0	18:0	18:1t	18:1c	18:2 ct+tt	18:2 cc	18:3	Total trans GC	IR
2	5.3	5.1	30.2	31.2	1.3	23.3	3.6	31.5	33.3
5	5.3	5.1	30.7	30.8	1.2	22.7	3.6	31.9	31.9
7	5.4	5.0	30.9	31.7	1.4	22.5	3.2	32.3	33.4
10	6.0	5.3	30.1	30.8	0.3	23.7	3.7	30.4[b]	29.9
11	5.4	5.2	30.8	33.3	0.6	21.8	2.8	31.4	33.7
12	5.5	5.1	30.7	30.6	1.0	23.4	3.6	31.7	36.8
13	5.3	5.1	30.5	30.3	1.2	23.3	3.8	31.7	—[c]
$\bar{x}$, %	5.4	5.1	30.6	31.2	1.0	23.0	3.5	31.6	33.2
SD	0.25	0.10	0.30	1.01	0.40	0.66	0.35	0.59	2.27
CV, %	4.6	1.9	1.0	3.2	40.4	2.9	10.0	1.9	6.8
Known value, %	5.2	5.1	30.7	29.9	1.1	24.0	4.0	31.8	31.8
Rec., %	103.8	100.0	99.7	104.3	90.9	95.8	87.5	99.4	104.4

[a]See Table 2, footnote a.
[b]Dixon outlier at 95% confidence level.
[c]No IR analysis.

Table 10. GC analysis: margarine methyl ester sample run in duplicate (ct-4-A and ct-4-B)

Statistic	Fatty acid[a] 16:0	18:0	18:1t	18:1c	18:2 ct+tt	18:2 cc	18:3	Total trans GC[b]	IR[c]
$\bar{x}$, %	11.0	9.2	21.7	26.0	0.6	27.9	3.2	22.3	22.9
SD_o[d]	0.41	0.10	0.13	0.23	0.06	0.27	0.11	0.12	—
SD_x[e]	0.52	0.27	0.38	1.01	0.52	0.99	0.43	0.50	1.20
CV_o,[f] %	3.74	1.05	0.58	0.90	9.13	0.99	3.37	0.52	—
CV_x,[g] %	4.69	2.95	1.73	3.90	85.61	3.55	13.37	2.23	5.24

[a]See Table 2, footnote a.
[b]$N = 13$.
[c]$N = 11$.
[d]Repeatability standard deviation, i.e., within-laboratory variation, reflecting random error.
[e]Reproducibility standard deviation, i.e., between-laboratory variation, reflecting variation arising from different analysts, apparatus, and laboratories.
[f]Ratio of SD_o to $\bar{x}$ times 100 ($100\ SD_o/\bar{x}$).
[g]Ratio of SD_x to $\bar{x}$ times 100 ($100\ SD_x/\bar{x}$).

columns, whereas 8–10 components were observed with the packed column.

One of the collaborators pointed out that resolution can be lost while using the OV-275 packed column. However, replacing the first 6 in. of the column with fresh coated packing material completely regenerated the column efficiency. Another collaborator reported that columns are prepared in his laboratory and are renewed at least every 3 months. Columns in use are disconnected and stored overnight at 120°C under a stream of pure nitrogen.

The statistical data from the collaborative study are in excellent agreement with data from a collaborative study of the AOAC-IUPAC GC method for determination of methyl esters of fatty acids (3, 4). Coefficients of variation (CVs) for components in the range of 4–40% were comparable (2–6% for soybean oil methyl esters (AOAC-IUPAC study) as well as for margarine methyl esters (this study)). Good recoveries of total trans acids as well as individual components were also observed in this study (Table 4; methyl ester standards); however, recoveries of total trans acids by IR were always greater than 100% and higher than recoveries of total trans acids by GC.

Between-laboratory as well as within-laboratory CVs were consistently better for the determination of total trans acids

Table 11. GC analysis: margarine methyl ester sample run in duplicate (ct-4-A and ct-6)

Statistic	Fatty acid[a]							Total trans	
	16:0	18:0	18:1t	18:1c	18:2 ct+tt	13:2 cc	18:3	GC[b]	IR[c]
$\bar{x}$, %	10.9	9.1	21.7	26.0	0.6	27.9	3.2	22.3	22.7
$SD_o{}^d$	0.26	0.11	0.16	0.32	0.14	0.30	0.09	0.19	0.67
$SD_x{}^e$	0.49	0.24	0.35	1.07	0.53	0.82	0.40	0.50	1.24
$CV_o{}^f$ %	2.42	1.25	0.76	1.24	23.1	1.08	2.79	0.85	2.93
$CV_x{}^g$ %	4.52	2.63	1.62	4.13	86.2	2.94	12.56	2.22	5.45

[a]See Table 2, footnote a.
[b]N = 13.
[c]N = 11.
[d]Repeatability standard deviation, i.e., within-laboratory variation, reflecting random error.
[e]Reproducibility standard deviation, i.e., between-laboratory variation, reflecting variation arising from different analysts, apparatus, and laboratories.
[f] Ratio of SD_o to $\bar{x}$ times 100 (100 $SD_o/\bar{x}$).
[g]Ratio of SD_x to $\bar{x}$ times 100 (100 $SD_x/\bar{x}$).

Table 12. GC analysis: margarine methyl ester sample run in duplicate (ct-4-B and ct-6)

Statistic	Fatty acid[a]							Total trans[b]
	16:0	18:0	18:1t	18:1c	18:2 ct+tt	18:2 cc	18:3	
$\bar{x}$, %	11.0	10.2	21.7	25.9	0.6	27.9	3.2	22.4
$SD_o{}^c$	0.41	1.38	0.24	0.19	0.13	0.43	0.12	0.23
$SD_x{}^d$	0.53	1.38	0.41	1.01	0.54	0.93	0.41	0.50
$CV_o{}^e$ %	3.72	13.6	1.11	0.72	21.5	1.56	3.63	1.03
$CV_x{}^f$ %	4.80	13.6	1.87	3.90	86.2	3.34	12.62	2.25

[a]See Table 2, footnote a.
[b]N = 13.
[c]Repeatability standard deviation, i.e, within-laboratory variation, reflecting random error.
[d]Reproducibility standard deviation, i.e., between-laboratory variation, reflecting variation arising from different analysts, apparatus, and laboratories.
[e]Ratio of SD_o to $\bar{x}$ times 100 (100 $SD_o/\bar{x}$).
[f] Ratio of SD_x to $\bar{x}$ times 100 (100 $SD_x/\bar{x}$).

by GC vs IR. CV values were generally 2–3 times higher for total *trans* acids by IR. For example, the collaborative data for margarine methyl ester blind duplicates ct-4-A and ct-6 (Tables 3 and 6) yielded within- and between-laboratory CVs for total *trans* acids by IR that are about 2.5 and 3.5 times higher, respectively, than the values for total *trans* acids by GC (Table 11). Similar CV values were obtained for analysis of ct-4 in duplicate and analysis of blind duplicates ct-4 and ct-6 (Tables 10 and 12).

Recommendation

It is recommended that the packed column GC method for determining *trans* unsaturation in margarine be adoped official first action to provide an alternative procedure to the AOAC IR method (2).

Acknowledgments

References

(1) Perkins, E. G., McCarthy, T. P., O'Brien, M. A., & Kummerow, F. A. (1977) *J. Am. Oil Chem. Soc.* **54,** 279–281

(2) *Official Methods of Analysis* (1980) 13th Ed., AOAC, Arlington, VA, secs **28.075–28.080**

(3) Firestone, D., & Horwitz, W. (1979) *J. Assoc. Off. Anal. Chem.* **62,** 709–721

(4) *Official Methods of Analysis* (1980) 13th Ed., AOAC, Arlington, VA, secs **28.057–28.065**

VITAMINS AND OTHER NUTRIENTS

Comparison of Interlaboratory Variation in Amino Acid Analysis and Rat Growth Assays for Evaluating Protein Quality

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Estimates of inter- and intralaboratory variation of protein efficiency ratio (PER), relative PER (RPER), net protein ratio (NPR), relative NPR (RNPR), and nitrogen utilization (NU) were compared with those of amino acid analysis in the same batches of 7 protein sources (ANRC casein, egg white solids, minced beef, soy assay protein, rapeseed protein concentrate, pea flour, and whole wheat flour). Interlaboratory variation (estimated as between-laboratories coefficients of variation, CV) of NPR and RNPR (up to 6.0%) was lower than that of PER (up to 20.2%) and RPER (up to 18.5%). The interlaboratory determination of NPR and RNPR was also more reproducible than that of most essential amino acids (CV up to 10.0%), especially tryptophan (CV up to 23.7%), cystine (CV up to 17.6%), and methionine (CV up to 16.1%). Intralaboratory variation (estimated as within-laboratories CV) of amino acid analysis (up to 4.7%), however, was comparable to that of protein quality indices in most protein sources (up to 6.0%). The significant ($P < 0.01$) positive correlations ($r = 0.68$–0.74) between amino acid scores and protein quality indices based on rat growth were further improved when amino acid scores were corrected for digestibility of protein ($r = 0.73$–0.78) or individual amino acids ($r = 0.79$–0.82).

Sarwar et al. (1) studied the inter- and intralaboratory variation in amino acid analysis of 7 protein sources (casein, egg white solids, minced beef, soy assay protein, rapeseed protein concentrate, pea flour, and whole wheat flour). The 7 protein sources were also tested in a collaborative rat growth study for the determination of protein efficiency ratio (PER), relative PER (RPER), net protein ratio (NPR), relative NPR (RNPR), and nitrogen utilization (NU) (2). The protein sources used in both investigations (1, 2) were from the same batches and were of similar composition.

PER is the official method for assessing quality of food proteins in the United States and Canada, but this test has been criticized for not properly crediting protein used in maintenance and for lack of precision, poor reproducibility, and high cost (3). In the scientific community, there is considerable support to replace the PER method with an appropriate in vitro method based on amino acid analysis (4). Therefore, it was of interest to compare the estimates of interlaboratory variations for amino acid analysis and rat growth methods for evaluating protein quality of foods.

In the present investigation, the PER, RPER, NPR, RNPR, and NU data of Sarwar et al. (2) were re-analyzed to calculate the between- and within-laboratory coefficients of variation

(CV) in the same manner as was done in the collaborative amino acid study (1). The newly calculated variability estimates for PER, RPER, NPR, RNPR, and NU were compared with those reported for amino acids (1).

METHODS

The PER, RPER, NPR, RNPR, and NU data of Sarwar et al. (2) for 7 protein sources [casein, minced beef, soy assay protein (SAP), pea flour, whole wheat flour (WW), rapeseed protein concentrate (RPC), egg white solids (EW)] were re-analyzed in the present study. The arithmetic means for each protein source were computed for PER, RPER, NPR, RNPR, and NU. Analysis of variance techniques were used to obtain measures of between-laboratories and within-laboratories variability (5) for each protein quality index within each protein source. The between-laboratories standard errors (SE) and CV and within-laboratories CV were calculated according to the previously described (1) formulas:

$$\text{Between-laboratories SE} = \sqrt{\frac{\hat{\sigma}_A^2}{7}}$$

where $\hat{\sigma}_A^2$ is the between-laboratories mean square from the analysis of variance.

$$\text{Between-laboratories CV} = \frac{\hat{\sigma}_A}{\sqrt{7}\,(\text{mean})} \times 100$$

$$\text{Within-laboratories CV} = \frac{\hat{\sigma}_W}{\sqrt{7}\,(\text{mean})} \times 100$$

where $\hat{\sigma}_W$ is the square root of the within-laboratories mean squares, $\hat{\sigma}_W^2$.

Results and Discussion

Means and SE and CV values for 5 protein quality indices and 10 amino acids in the 7 protein sources are given in Tables 1–7. The protein quality results were calculated as the average of 7 determinations, whereas the amino acid results were calculated as the average of 2 determinations (1). Obviously, one can make either method more precise by averaging more determinations.

In casein, the between-laboratories variation (estimated as CV) for most amino acids (4.2–7.1%) except cystine (17.6%) and tryptophan (14.3%) was comparable to the variation for protein quality indices (3.0–7.6%, Table 1). RNPR had the lowest between-laboratories CV value. The within-laboratories variation for amino acids (0.6–2.6%) was somewhat lower than that for protein quality indices (2.2–3.4%, Table 1).

In egg white, the between-laboratories CV values for amino acids (7.7–19.7%) were higher than those for protein quality

Table 1. Means, standard errors (SE), and coefficients of variation (CV) of protein quality indices and amino acid analysis of casein

| Index[a] | Mean | Between laboratories | | Within laboratories |
		SE	CV, %	CV, %
PER	3.14	0.24	7.6	3.4
RPER	78.00	4.39	5.6	2.5
NPR	4.55	0.25	5.5	3.3
RNPR	86.00	2.56	3.0	2.2
NU	4.83	0.34	7.0	3.4
Amino acid (g/16 g N)[b]				
lie	5.36	0.26	4.8	1.3
Leu	10.16	0.44	4.3	0.7
Lys	8.44	0.42	5.0	0.6
Met	3.02	0.13	4.2	0.8
Cys	0.47	0.08	17.6	2.6
Phe	5.47	0.39	7.1	1.5
Tyr	6.04	0.42	6.9	1.2
Thr	4.64	0.32	7.0	1.5
Trp	1.31	0.19	14.3	1.1
Val	6.85	0.30	4.4	1.4

[a]PER = protein efficiency ratio; RPER = relative protein efficiency ratio; NPR = net protein ratio; RNPR = relative net protein ratio; NU = nitrogen utilization. Formulas for calculating these indices have been previously reported (2).
[b]The data for amino acids were taken from Sarwar et al. (1). Ile = isoleucine; Leu = leucine; Lys = lysine; Met = methionine; Cys = cystine; Phe = phenylalanine; Tyr = tyrosine; Thr = threonine; Trp = tryptophan; Val = valine.

Table 2. Means, standard errors (SE), and coefficients of variation (CV) of protein quality indices and amino acid analysis of egg white

| Index[a] | Mean | Between laboratories | | Within laboratories |
		SE	CV, %	CV, %
PER	3.71	0.20	5.4	2.8
RPER	91.00	3.06	3.4	2.6
NPR	5.08	0.22	4.4	2.0
RNPR	95.00	1.46	1.5	1.6
NU	5.43	0.27	5.0	1.9
Amino acid (g/16 g N)[b]				
Ile	5.28	0.59	11.2	2.2
Leu	8.76	0.74	8.4	1.7
Lys	6.98	0.62	8.8	2.5
Met	3.83	0.32	8.4	1.8
Cys	2.81	0.31	11.0	1.7
Phe	6.21	0.57	9.3	1.0
Tyr	4.40	0.34	7.7	2.5
Thr	4.68	0.46	9.9	2.7
Trp	1.46	0.29	19.7	1.5
Val	6.78	0.64	9.5	2.0

[a,b]See Table 1.

Table 3. Means, standard errors (SE), and coefficients of variation (CV) of protein quality indices and amino acid analysis of beef

| Index[a] | Mean | Between laboratories | | Within laboratories |
		SE	CV, %	CV, %
PER	3.36	0.24	7.2	2.7
RPER	83.00	2.87	3.4	2.4
NPR	4.83	0.26	5.3	2.2
RNPR	91.00	3.24	3.6	1.9
NU	5.16	0.32	6.2	2.3
Amino acid (g/16 g N)[b]				
Ile	4.18	0.14	3.4	0.8
Leu	7.75	0.46	5.9	1.4
Lys	7.94	0.75	9.5	1.2
Met	2.25	0.24	10.5	1.4
Cys	1.02	0.13	12.8	2.7
Phe	3.88	0.33	8.6	2.0
Tyr	3.14	0.22	7.1	2.1
Thr	4.21	0.17	4.0	2.7
Trp	0.99	0.17	16.9	2.6
Val	4.54	0.25	5.5	2.3

[a,b]See Table 1.

Table 4. Means, standard errors (SE), and coefficients of variation (CV) of protein quality indices and amino acid analysis of soy assay protein

| Index[a] | Mean | Between laboratories | | Within laboratories |
		SE	CV, %	CV, %
PER	1.60	0.21	13.1	6.0
RPER	39.00	5.14	13.0	5.2
NPR	2.74	0.10	3.7	4.0
RNPR	51.00	3.11	6.0	3.0
NU	2.79	0.19	7.0	4.4
Amino acid (g/16 g N)[b]				
Ile	4.72	0.16	3.3	2.5
Leu	8.51	0.40	4.7	2.0
Lys	6.34	0.37	5.8	1.9
Met	1.24	0.14	11.3	4.5
Cys	1.19	0.14	11.4	2.4
Phe	5.52	0.39	6.9	1.4
Tyr	4.04	0.18	4.4	2.0
Thr	3.34	0.34	8.8	1.1
Trp	1.14	0.27	23.7	3.7
Val	4.91	0.33	6.8	1.4

[a,b]See Table 1.

Table 5. Means, standard errors (SE), and coefficients of variation (CV) of protein quality indices and amino acid analysis of rapeseed protein concentrate

| Index[a] | Mean | Between laboratories | | Within laboratories |
		SE	CV, %	CV, %
PER	3.29	0.22	6.9	3.4
RPER	81.00	2.18	2.7	2.6
NPR	4.59	0.25	5.5	2.7
RNPR	87.00	2.99	3.4	2.0
NU	4.90	0.29	5.9	2.7
Amino acid (g/16 g N)[b]				
lie	4.'6	0.38	9.1	4.1
Leu	7.84	0.41	5.2	0.9
Lys	5.70	0.33	5.8	2.9
Met	1.97	0.25	12.8	1.7
Cys	2.46	0.43	17.5	4.3
Phe	4.24	0.40	9.5	1.8
Tyr	2.82	0.38	13.5	3.9
Thr	4.19	0.35	8.3	3.6
Trp	1.50	0.23	15.2	2.9
Val	5.24	0.35	6.7	2.5

[a,b]See Table 1.

Table 6. Means, standard errors (SE), and coefficients of variation (CV) of protein quality indices and amino acid analysis of pea flour

| Index[a] | Mean | Between laboratories | | Within laboratories |
		SE	CV, %	CV, %
PER	1.56	0.31	19.7	4.9
RPER	39.00	6.10	15.8	4.4
NPR	2.88	0.13	4.5	4.5
RNPR	54.00	2.56	4.7	3.4
NU	2.89	0.30	10.3	4.1
Amino acid (g/16 g N)[b]				
Ile	4.27	0.34	7.9	4.7
Leu	7.67	0.55	7.1	3.1
Lys	7.68	0.52	6.8	2.8
Met	1.11	0.18	16.1	4.7
Cys	1.51	0.21	14.1	4.3
Phe	4.99	0.49	9.8	2.0
Tyr	3.50	0.57	16.4	3.1
Thr	4.17	0.22	5.4	1.7
Trp	0.87	0.16	18.5	3.0
Val	4.96	0.50	10.0	2.3

[a,b]See Table 1.

Table 7. Means, standard errors (SE), and coefficients of variation (CV) of protein quality indices and amino acid analysis of wheat flour

| Index[a] | Mean | Between laboratories | | Within laboratories |
		SE	CV, %	CV, %
PER	0.95	0.19	20.2	9.1
RPER	24.00	4.33	18.5	8.6
NPR	2.35	0.12	5.1	4.3
RNPR	45.00	2.16	4.8	3.7
NU	2.29	0.17	7.6	4.7
Amino acid (g/16 g N)[b]				
Ile	3.34	0.24	7.2	2.2
Leu	6.85	0.31	4.6	1.7
Lys	2.66	0.19	7.3	2.7
Met	1.65	0.20	12.2	4.0
Cys	2.22	0.31	14.1	2.0
Phe	4.87	0.42	8.6	1.0
Tyr	2.91	0.42	14.5	4.5
Thr	2.93	0.28	9.7	1.5
Trp	1.12	0.25	22.4	2.7
Val	4.27	0.35	8.2	1.7

[a,b]See Table 1.

methods (1.5–5.4%, Table 2). RNPR gave the lowest between-laboratories variation. The within-laboratories CV values for most amino acids and protein quality indices were less than 3% (Table 2).

In beef, the between-laboratories variations for tryptophan (16.9%), cystine (12.8%), methionine (10.5%), and lysine (9.5%) were considerably higher than the variation for RPER and RNPR (3.4 and 3.6%), whereas the within-laboratories variations for most amino acids and protein quality methods were similar (less than 3%, Table 3).

In soy assay protein, the between-laboratories CV values for all amino acids (3.3–11.4%) except tryptophan (23.7%) were lower than those for RPER and PER (13.0 and 13.1%), whereas the within-laboratories CV values for most amino acids were lower than the values for any of the 5 protein quality methods (Table 4).

In rapeseed protein concentrate, the between-laboratories variability for most amino acids, especially cystine (17.5%) and tryptophan (15.2%), was higher than that for protein quality methods (2.7–6.9%, Table 5). The within-laboratories variations for most amino acids and protein quality indices were similar (Table 5).

In pea flour, the between-laboratories CV values for most amino acids were lower than those for PER and RPER but higher than those for NPR and RNPR (Table 6). The differences among the within-laboratories CV values for amino acids and protein quality methods were small (Table 6).

In wheat flour, the between-laboratories variations for all amino acids (4.6–14.5%) except tryptophan (22.4%) were lower than the variations for RPER and PER (18.5 and 20.2%) but higher than those for RNPR and NPR (4.8 and 5.1%, Table 7). The within-laboratories variations for most amino acids were lower than those for any of the protein quality indices (Table 7).

In most protein sources, the interlaboratory variation (estimated as between-laboratories CV) was less than 10% for all amino acids except tryptophan, methionine, and cystine, as well as for NPR, RNPR, and NU (Tables 1–7). The interlaboratory variations (estimated as between-laboratories CV) for tryptophan (14.3–23.7%), cystine (11.0–17.6%), and methionine (up to 16.5%) were relatively large. In 4 protein sources (casein, egg white, beef, and rapeseed concentrate), the intralaboratory variation for PER and RPER was less than 10%, but the variation was large (13.0–20.2%) in the

remaining poor quality protein sources (soy assay protein, pea flour, and wheat flour). In most cases, the intralaboratory variations for amino acids and protein quality indices were less than 3 and 5%, respectively (Tables 1–7).

Theoretically, amino acid score is the best method for evaluating protein quality, but its validity is limited by large interlaboratory error for certain essential amino acids, lack of a suitable method for assessing availability of amino acids, and uncertainty about human requirements for amino acids (6).

During the last few years, much progress has been made in overcoming obstacles to using amino acid score. This study demonstrated that the interlaboratory variation for those essential amino acids determined after hydrolysis with 6N HCl was comparable to that for NPR and RNPR, which were more reproducible than PER and RPER values (Tables 1–7). Further standardization of methods for the determination of tryptophan and sulfur amino acids (especially cystine) is required to reduce to acceptable levels the interlaboratory variation for these amino acids; such studies are currently under way.

Another major problem preventing widespread acceptance of amino acid score is the lack of suitable methods for estimating availability of amino acids (6). The available amino acid score (amino acid score corrected for true digestibility of individual amino acids, as determined by rat balance method), solves this problem (7).

A general consensus appears to be forming about human amino acid reference pattern. The FAO/WHO 1973 (8) and NRC 1980 (9) human scoring patterns are similar, but the difference in methionine + cystine requirements is significant (Table 8). An evaluation of a soy protein isolate-based infant formula, using the FAO/WHO 1973 (8) pattern, would indicate the need to supplement this formula with methionine, but use of the NRC 1980 (9) pattern would indicate no need for supplementation. Rat assays indicate that soy protein-based infant formulas have relatively poor quality and should be supplemented with methionine. Studies with infants, however, indicate that methionine supplementation is unnecessary and probably undesirable (12).

Pinada et al. (13) and Torun et al. (14) conducted extensive studies on essential amino acid requirements of children aged 21–27 months. Most of the estimated requirements were similar to values in the FAO/WHO 1973 (8) pattern, but the methionine + cystine value was almost identical to the value in the NRC 1980 (9) pattern. The NRC 1980 (9) pattern contains only 66% of the amount of methionine + cystine required by the rat (Table 9). If the NRC 1980 (9) pattern is appropriate for humans, then the rat is an inappropriate animal for evaluating protein quality for humans, especially of foods deficient in methionine + cystine. As Steinke (15) suggested, the use of a correction factor (based on differences in rat and human requirements for essential amino acids) to adjust rat protein quality values should be considered.

Because direct evaluation with humans is impractical, an appropriate alternative to evaluate protein quality would be the use of an amino acid score based on the most appropriate human amino acid pattern and corrected for digestibility of protein and/or bioavailability of amino acids (4). In reviewing the relationship between amino acid scores and rat bioassays, Pellett (16) reported a high correlation ($r = 0.84$) between the net protein utilization (NPU) and amino acid score corrected for protein digestibility (Score × D) of 71 food items or mixtures. Sarwar (7) determined amino acid-digestibility scores (scores corrected for true protein digestibility) and available amino acid scores for 17 foods (mainly mixtures) and reported

Table 8.   Comparison of human amino acid scoring patterns and amino acid requirements of the rat

Amino acid (mg/g protein)	Human amino acid scoring pattern		Young rat	
	FAO/WHO 1973 (8)	NRC 1980[a] (9)	Hegsted[b] (10)	NRC (11)
Isoleucine	40	42	36	42
Leucine	70	70	69	62
Lysine	55	51	60	58
Methionine + cystine	35	26	40	50
Phenylalanine + tyrosine	60	73	59	67
Threonine	40	35	43	42
Tryptophan	10	11	11	12
Valine	50	48	54	50

[a]Amino acid pattern for high quality proteins.
[b]Recalculated from Hegsted (10), lowest set of values.

Table 9.   RNPR, relative NU (RNU), RPER, amino acid scores (AAS), amino acid-digestibility scores (AADS), and available amino acid scores (AAAS)[a]

Diet	RNPR, %	RNU,[b] %	RPER, %	AAS, %	AADS, %	AAAS, %
Casein + Met[c]	100	100	100	100	100	100
Egg white (EW)	95	96	91	100	100	100
Minced beef	91	91	83	90	88	86
RPC[d]	87	86	81	97	92	89
Casein	86	85	78	100	100	100
Pea flour + Met	81	81	72	79	73	65
WW[e] + casein	79	79	74	100	98	91
WW + beef	79	78	73	90	84	78
WW + EW	77	77	72	94	90	85
SAP[f] + Met	70	68	64	100	98	77
WW + pea flour	66	63	54	90	83	77
WW + SAP	64	62	55	88	81	76
WW + RPC	63	61	51	82	76	70
WW + Lys[g]	55	52	42	79	74	70
Pea flour	54	51	39	79	70	64
SAP	51	49	39	93	89	83
WW	44	40	23	52	47	41

[a]Data for RNPR, NU, and RPER were taken from Sarwar et al. (2).
[b]RNU = (NU of test protein/NU of casein + Met) × 100.
[c]Met = methionine.
[d]RPC = rapeseed protein concentrate.
[e]WW = whole wheat flour.
[f]SAP = soy assay protein.
[g]Lys = lysine.

Table 10.   Correlation coefficients among protein quality indices noted in Table 9

Index[a]	AAS	AADS	AAAS	RNPR	RNU	RPER
AADS	0.98					
AAAS	0.93	0.95				
RNPR	0.68	0.73	0.79			
RNU	0.68	0.73	0.79	1.00		
RPER	0.74	0.78	0.82	0.99	0.99	

[a]AAS = amino acid score; AADS = amino acid-digestibility score; AAAS = available amino acid score; RNPR = relative net protein ratio; RNU = relative nitrogen utilization; RPER = relative protein efficiency ratio.

amino acid score, of the SAP diet, however, may not be entirely due to higher sulfur amino acid requirements of the growing rat compared with those for humans. In one laboratory, a soybean protein isolate (having similar amino acid composition as SAP) was found to have a 65% RNPR. This may suggest the presence of antinutritional factor(s) in the SAP sample used in the present investigation.

The significant positve $(P < 0.01)$ correlations between available amino acid score and RNPR, RNU, or RPER $(r = 0.79–0.82)$ were higher than the correlations between amino acid scores and RNPR, RNU, or RPER $(r = 0.68–0.74)$ as well as between amino acid-digestibility scores and RNPR, RNU, or RPER $(r = 0.73–0.78)$. This investigation (Tables 9 and 10) suggested that amino acid scores corrected for digestibility of individual amino acids or total protein are better predictors of protein quality than are uncorrected scores. Similar conclusions about the desirability of correcting amino acid scores for lack of total nitrogen digestibility and/or amino acid availability were recently made at the third session of the Codex Committee on Vegetable Proteins (17).

REFERENCES

(1) Sarwar, G., et al. (1983) J. Food Sci. 48, 526–531
(2) Sarwar, G., et al. (1984) J. Assoc. Off. Anal. Chem. 67, 976–981.
(3) McLaughlan, J. M., et al. (1980) J. Assoc. Off. Anal. Chem. 63, 462–467
(4) Harper, A. E. (1981) in Protein Quality in Humans: Assessment and In Vitro Estimation, C. E. Bodwell, J. S. Adkins, & D. T. Hopkins (Eds), AVI Publishing Co., Inc., Westport, CT, pp. 417–420
(5) Youden, W. J., & Steiner, E. H. (1975) Statistical Manual of the AOAC, ACAC, Arlington, VA
(6) Codex Alimentarius Commission Document CX/VP 82/3 (1982) Report of Ad Hoc Working Group on Protein Quality Measurement (coordinated and prepared by J. M. McLaughlan), Food and Agriculture Organization, Rome; World Health Organization, Geneva
(7) Sarwar, G. (1984) J. Assoc. Off. Anal. Chem. 67, 623–626
(8) FAO/WHO (1973) Energy and Protein Requirements, Food and Agriculture Org. Nutr. Rep. Ser. No. 52, Rome; World Health Org. Techn. Rep. Ser. No. 522, Geneva
(9) National Research Council (1980) Recommended Dietary Allowances, NRC, National Academy of Sciences, Washington, DC
(10) Hegsted, D. M. (1973) Protein in Human Nutrition, in J. W. G. Porter & A. B. Rolls (Eds), Academic Press, New York, NY, pp. 275–291
(11) National Research Council (1978) Nutrient Requirements of Laboratory Animals, 3rd Rev. Ed., NRC, National Academy of Sciences, Washington, DC
(12) Scrimshaw, N. S. (1981) in Protein Quality in Humans: Assessment and In Vitro Estimation, C. E. Bodwell, J. S. Adkins, & D. T. Hopkins (Eds), AVI Publishing Co., Inc., Westport, CT p. 371

highly significant positive correlations $(r = 0.92)$ between RNPR and available amino acid score or amino acid-digestibility score. Both Pellett (16) and Sarwar (7) used the FAO/WHO 1973 (8) pattern for calculating scores, which also suits the rat amino acid requirement (Table 8). In the present investigation, therefore, scores were calculated by using the NRC 1980 (9) scoring pattern (Table 9). The previously reported data on amino acid contents and true digestibility of protein and individual amino acids (7) were used in these calculations.

In most cases, amino acid scores were considerably higher than corresponding RPER (up to 40 units) and RNPR or RNU (up to 32 units) values (Table 9). The differences between amino acid-digestibility scores or available amino acid scores and corresponding RPER, RNPR, or RNU values, however, were not as large. In all diets except SAP, the amino acid-digestibility scores and available amino acid scores were up to 30 and 19 units higher than corresponding RNPR or RNU values, respectively. The lower ratings by the RNPR and RNU methods (which credit protein used for both growth and maintenance) compared with the available amino acid scores would reflect the higher essential amino acid requirements of the growing rat compared with those for humans. The RNPR or RNU value, substantially lower than available

(13) Pineda, O., Torun B., Viteri, F. E., & Arroyave, G. (1981) in *Protein Quality in Humans: Assessment and In Vitro Estimation*, C. E. Bodwell, J. S. Adkins, & D. T. Hopkins (Eds), AVI Publishing Co., Inc., Westport, CT, pp. 29–42

(14) Torun, B., Pineda, O., Vieri, F. E., & Assoyave, G. (1981) in *Protein Quality in Humans: Assessment and In Vitro Estimation*, C. E. Bodwell, J. S. Adkins, & D. T. Hopkins (Eds), AVI Publishing Co., Inc., Westport, CT, pp. 374–393

(15) Steinke, F. H. (1979) in *Soy Protein and Human Nutrition*, H. L. Wilcke, D. T. Hopkins, & D. H. Waggle (Eds), Academic Press, New York, NY, pp. 307–312

(16) Pellett, P. L. (1978) *Food Technol.* **32**, 61–79

(17) Codex Alimentarius Commission Document CX/VP 85/30 (1984) Report of the third session of the Codex Committee on Vegetable Proteins, Ottawa, 6–10 February 1984, Food and Agriculture Organization, Rome; World Health Organization, Geneva

Comparison of Carr-Price Analysis and Liquid Chromatographic Analysis for Vitamin A in Fortified Milk

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The determination of the vitamin A concentration in fortified milk was compared using Carr-Price analysis and liquid chromatography (LC). Carr-Price analysis required saponification of the sample with alcoholic potassium hydroxide, extraction with ether, and colorimetry with antimony trichloride in chloroform. LC analysis required hexane extraction of a 71% alcohol–sample solution and centrifugation at 2000 rpm. A 100 μL aliquot of the extract was analyzed on a LiChrosorb Si-60, 5 μm column, using an ethyl ether–hexane (2 + 98) mobile phase and detection at 313 nm. Each method was statistically evaluated for precision and sample-to-sample reproducibility. The LC extraction procedure was examined for efficiency. Each LC value was divided by the Carr-Price value obtained for the same sample; an average value of 0.975 with a coefficient of variation of 6.90% was obtained. It was concluded that the procedures were statistically equivalent.

Milk that is partially or wholly defatted is fortified with vitamin A; it is the main source of vitamin A for most people. As a producer, this laboratory must monitor the vitamin A levels added to milk to verify that they are within U.S. Food and Drug Administration limits. For several years, the analytical method of choice was a non-AOAC, Carr-Price colorimetric procedure (1). Because of its chemical hazards, end product instability, and intensive labor demands, an alternative method of analysis was desired.

It was determined that any alternative method had to be simple, less intensive, less hazardous, reproducible, and comparable to the Carr-Price procedure. A fluorometric method (2) was initially considered, but equipment availability dictated a preference for a liquid chromatographic (LC) procedure. Two reverse phase LC procedures (3, 4) were considered, but only one was tested (3). This procedure was reproducible and less hazardous than the Carr-Price method. Although simpler, this procedure still required saponification, several extractions, and an evaporation and redissolving step that could possibly produce varying results. Before we could perform a comparison study, the same authors published another procedure using normal phase LC (5). This latter procedure was reproducible, less hazardous, very simple, and less time-consuming, requiring less than 1 h for analysis of a single sample. We chose this normal phase LC method for comparison with the Carr-Price procedure.

Carr-Price Method

Apparatus and Reagents

(a) *Spectrophotometer.*—Coleman Model 620 set at 620 nm, or equivalent, with 25 mL cuvets.

(b) *Alcoholic potassium hydroxide.*—Dissolve 25 g KOH in 50 mL water, cool, and add 350 mL ethanol.

(c) *Petroleum ether.*—Reagent grade, bp 35–60°C.

(d) *Sodium sulfate anhydrous.*—Reagent grade.

(e) *Ethyl ether.*—Reagent grade.

(f) *Carr-Price reagent.*—Transfer entire contents of 113 g (¼ lb) unopened bottle of reagent grade antimony trichloride to 1 L screw-cap Erlenmeyer flask. Crystals should be translucent without any color or decomposition products present. Add 500 mL chloroform, warm, and stir to dissolve. Cool and add 30 mL acetic anhydride. Store cool and in the dark.

Preparation of Sample

Mix milk well before sampling. Pipet 30 mL milk and 20 mL alcoholic KOH into 150 mL beaker, cover with watch glass, and immerse in 70°C water bath 30 min, mixing occasionally. Cool solution and quantitatively transfer to 125 mL separatory funnel with ca 10–20 mL alcohol.

Add 35 mL ethyl ether to separatory funnel and shake vigorously 5 min. Add 5 mL petroleum ether to funnel, swirl, and let layers separate. Transfer aqueous layer to second separatory funnel and repeat ethyl ether extraction for 2 min. Add 5 mL petroleum ether to funnel, swirl, and let layers separate. Discard aqueous layer and quantitatively transfer contents of second separatory funnel to the first with 5–10 mL ethyl ether.

Add 25–30 mL water to separatory funnel and gently mix to remove KOH from ether phase. Discard aqueous layer and repeat water wash until wash is neutral to phenolphthalein indicator.

Evaporate ether phase to less than 40 mL with nitrogen, discard any residual water in funnel, and quantitatively transfer ether solution to 50 mL volumetric flask. Dilute to volume with petroleum ether. Add ca 5 g anhydrous Na_2SO_4 and shake. A clear solution will result.

Preparation of Standard

Weigh ca 100 mg USP reference standard (vitamin A acetate in oil, capsule form, USP Reference Standards, U.S. Pharmacopeidal Convention, Inc., 12601 Twinbrook Pkwy, Rockville, MD 20852) to nearest 0.1 mg in 150 mL beaker. Add 30 mL water and 20 mL alcoholic KOH and proceed as in *Preparation of Sample,* starting " . . . cover with watch glass . . . ." Transfer contents of separatory funnel to 100 mL volumetric flask instead of 50 mL flask. Dilute this solution 1:100 to obtain vitamin A concentration of ca 1 IU/mL. Calculate exact concentration from weight of standard used (100 mg = 10 276 IU vitamin A).

Preparation of Standard Curve

Pipet 5, 10, 15, and 20 mL standard solution into clean 25 mL cuvets. Evaporate ether under nitrogen in warm water,

just to dryness, and immediately dissolve residue in 2 mL chloroform. Place 2 mL chloroform into clean cuvet for use as blank. Add 10 mL Carr-Price reagent from rapid delivery pipet to blank cuvet. Immediately place in spectrophotometer and set absorbance to zero. Repeat addition of Carr-Price reagent for each standard level. After addition of reagent, immediately place cuvet in spectrophotometer and read maximum absorbance. An unvarying colorimetry technique is essential because blue complex is unstable and dissipates quickly. Plot concentration against absorbance to obtain standard curve. Alternatively, calculate slope for use in sample calculations.

Sample Analysis

Pipet 10 mL sample into 25 mL cuvet. Evaporate ether under nitrogen in warm water, just to dryness, and immediately redissolve residue in 2 mL chloroform. Add 10 mL Carr-Price reagent, place cuvet in spectrophotometer, and read maximum absorbance as in *Preparation of Standard Curve.* From standard curve or slope, calculate the IU vitamin A in cuvet.

Calculations

Calculate vitamin A level in IU/quart milk as shown in the equation:

$$(IU/6) \times 946 = IU/qt \text{ milk}$$

where IU = IU content in cuvet; 6 = equivalent mL milk in cuvet; and 946 = total mL milk in 1 quart.

Liquid chromatographic Method

Apparatus and Reagents

(a) *Liquid chromatograph.*—Waters Model 6000A pump fitted with Rheodyne Model 7125 injector and 100 μL loop; E. Merck LiChrosorb Si-60, 5 μm column, 250 × 4.6 mm id; Waters Model 440 absorbance detector, wavelength 313 nm; and Hewlett-Packard Model 3390A integrator.

(b) *Wet hexane.*—Add 200 mL LC grade water to 4 L filtered (0.2 μm) hexane, mix, and let stand 24 h.

(c) *Mobile phase.*—Mix 49 parts wet hexane, 49 parts hexane, and 2 parts ethyl ether. Prepare fresh daily.

(d) *Cleaning mobile phase.*—25% ethyl ether in hexane.

Preparation of Standard

Dissolve ca 30 mg (weigh to nearest 0.1 mg) *trans*-retinyl palmitate (crystalline type IV synthetic, Sigma Chemical Co., St. Louis, MO 63178) with hexane in 100 mL volumetric flask and dilute to volume. Calculate IU/mL content from assayed value. Make serial dilutions to obtain working standard of ca 1 IU/ml.

Extraction of Milk

Mix sample thoroughly. Pipet 2.0 mL milk and 5.0 mL ethanol into 25 mL screw-cap vial, mix, and let stand 5 min. Pipet 5.0 mL hexane into vial and mix vigorously on vortex mixer 30 s. Let mixture stand 2 min. Repeat mixing and standing twice more. Pipet 3 mL water into tube, cap, and invert tube to mix. Centrifuge tube 10 min at 2000 rpm.

Determination of Retinyl Palmitate

Set flow rate of mobile phase to 2 mL/min and inject 100 μL working standard. Adjust detector and integrator conditions to 50% full scale deflection with standard. Calibrate

integrator on the basis of amount of vitamin A palmitate injected (1.0 IU/mL standard = 0.1 IU injected). Two peaks may be detected. Calibration is based on large *(trans)* peak.

Set multiplication factor of the integrator to 23 650 and inject 100 μL sample. Integrator will report results in IU vitamin A/quart milk.

Reinject standard solution after every 4 sample injections to verify constant response, and recalibrate if necessary. After every 16–20 samples, wash column 10–15 min with 25% ether in hexane cleaning mobile phase and then with mobile phase until retention time of the standard returns to normal.

Discussion

The Carr-Price method is the accepted method of vitamin A analysis in milk. This work statistically compared the LC method with the Carr-Price analysis for precision, sample reproducibility, and final value. A further objective was to determine if the LC extraction was as complete as described in the literature or if further extractions were necessary.

Extraction Efficiency

To verify extraction efficiency of the LC method, experimental samples of skim, 1%, and 2% milk were re-extracted after the initial extract was removed. The re-extraction was done with 2 solvents: hexane spiked with 0.94 IU retinyl palmitate/mL, and hexane unspiked. We reasoned that if the extraction were not complete, but in equilibrium, a significant decrease in the IU level would occur with spiked hexane. Similarly, using unspiked hexane, any unextracted retinyl palmitate would be recovered by re-extraction.

Very little change in the IU level was observed for re-extraction with spiked hexane. Four samples were re-extracted, and an average value of 0.95 IU/mL was obtained. Compared with the initial spike level of 0.94 IU/mL, the increase in detectable retinyl palmitate is statistically insignificant.

Five samples were re-extracted using unspiked hexane. A constant recovery value of 0.003 IU/mL was observed. This is equivalent to 71 IU/qt milk. Initially, it seemed that a significant, observable amount of vitamin A was still in the aqueous milk solution, but further examination of the data revealed that all recovered values were the same. This is unusual, because the observed values of vitamin A in each sample were not the same, indicating that the recovered vitamin A came from a source other than the aqueous extract.

We examined the re-extraction procedure and noted that all initial extracts of the samples were removed identically, without assurance that residual extracted vitamin A did not adhere to the inside of the vials. This would explain the unvarying recovery results for re-extraction with unspiked hexane. On the basis of the above observations, the recovery was considered complete after the initial extraction.

Precision

The Carr-Price and LC methods were examined for their precision for a single analysis. Each extract was analyzed 3 times and an average (X), standard deviation (SD), and coefficient of variation (CV) were determined (Table 1). Standard deviations for the Carr-Price method ranged from 8.00 to 155.24% and the coefficients of variation ranged from 0.29 to 5.83%. For the LC method, SD values ranged from 4.73 to 51.87%, and CV values ranged from 0.23 to 4.97%.

Each method was also examined for sample precision. Each sample was analyzed in triplicate and an average, standard deviation, and coefficient of variation were computed (Table 2). For the Carr-Price method, SD values ranged from

Table 1. Analysis precision for Carr-Price (CP) and LC determination of vitamin A in milk

Sample	CP			LC		
	IU/qt	SD	CV, %	IU/qt	SD	CV, %
Skim 1a	2060	21.13	1.03	2142	21.6	1.01
Skim 1b	2167	25.42	1.18	2182	25.3	1.16
Skim 1c	2113	62.91	1.98	2102	4.9	0.23
Skim 2a	1876	78.12	4.16	1731	7.02	0.41
Skim 2b	1864	43.12	2.31	1774	8.54	0.48
Skim 2c	1890	38.07	2.01	1720	8.08	0.47
1% 1a	2801	8.00	0.29	2996	30.05	1.02
1% 1b	2907	143.80	4.95	2897	17.8	0.61
1% 1c	2958	140.53	4.75	2974	24.0	0.81
1% 2a	2502	121.50	4.86	2677	31.00	1.95
1% 2b	2536	25.87	1.02	2664	51.38	1.09
1% 2c	2513	67.16	2.67	2652	29.00	1.23
1% 3a	1139	20.66	1.81	1043	51.87	4.97
1% 3b	1126	35.09	3.16	1027	22.03	2.15
1% 3c	1195	10.00	0.84	1053	16.92	1.61
1.5% 1a	2974	155.24	5.22	2846	43.78	1.54
1.5% 1b	3167	48.18	1.52	2720	27.40	1.01
1.5% 1c	3052	84.69	2.77	2853	15.31	0.54
2% 1a	1683	62.18	3.69	1717	29.4	1.71
2% 1b	1825	75.80	4.15	1635	5.69	0.35
2% 1c	1761	36.59	2.08	1616	4.73	0.29
2% 2a	2477	73.50	2.97	2683	32.87	1.23
2% 2b	2432	60.32	2.48	2574	29.46	1.14
2% 2c	2513	146.45	5.83	2733	17.62	0.64
2% 3a	2376	66.94	2.82	2138	23.59	1.10
2% 3b	2304	103.04	4.47	2246	41.24	1.81
2% 3c	2428	25.89	1.07	2134	38.55	1.81

Table 2. Reproducibility within a single sample for Carr-Price (CP) and LC determinations

Sample	CP			LC		
	IU/qt	SD	CV, %	IU/qt	SD	CV, %
Skim 1	2113	53.50	2.53	2143	38.02	1.77
Skim 2	1877	13.01	0.69	1742	28.53	1.64
1% 1	2889	80.09	2.78	2956	51.98	1.76
1% 2	2517	17.35	0.69	2664	12.50	0.47
1% 3	1153	36.67	3.18	1041	13.11	1.26
1.5% 1	3064	97.09	3.17	2806	74.84	2.67
2% 1	1756	71.11	4.05	1656	53.67	3.23
2% 2	2474	40.58	1.64	2663	81.30	3.05
2% 3	2369	62.27	2.63	2173	63.54	2.92

13.01 to 97.09, and CV values ranged from 0.69 to 4.05. For the LC method, SD values ranged from 12.50 to 81.30, and CV values ranged from 0.47 to 3.23.

These data indicate that the LC method has greater reproducibility than the Carr-Price method. The range of deviation is much less for the LC method: less than half of that found by the Carr-Price method for analysis precision and three-fourths of the Carr-Price range for sample precision. The coefficient of variation range is about 1% less for the LC method than that found for the Carr-Price in both analysis and sample precision.

Method Comparison

Because the LC method compared well with the Carr-Price method in precision, it was necessary only to compare results of both the Carr-Price and LC methods to judge the latter acceptable. The average values of each method were compared by dividing each LC value by the Carr-Price value. If the methods were equal, an ideal value of one would result.

The actual values ranged from 0.903 to 1.076 (Table 3) with an average value of 0.975, standard deviation of 0.067, and coefficient of variation of 6.90%. With less than 7% variation in results found between the methods, the LC procedure is considered comparable to the Carr-Price procedure.

Table 3. Comparison of LC analysis with Carr-Price analysis

Sample	LC, IU/qt	CP, IU/qt	LC/CP
Skim 1	2143	2113	1.014
Skim 2	1742	1877	0.928
1% 1	2956	2889	1.023
1% 2	2664	2517	1.058
1% 3	1041	1153	0.903
1.5% 1	2806	3064	0.916
2% 1	1656	1756	0.943
2% 2	2663	2474	1.076
2% 3	2173	2369	0.917

$\bar{X}$ = 0.975
SD = 0.067
CV = 6.90%

REFERENCES

(1) Association of Vitamin Chemists, Inc. (1951) *Methods of Vitamin Assay*, 2nd Ed., Interscience Publishers, Inc., New York, NY, pp. 26–35

(2) Thompson, J. N., Erodody, P., Maxwell, W. B., & Murray, T. K. (1972) *J. Dairy Sci.* **55**, 1077–1080

(3) Thompson, J. N., & Maxwell, W. B. (1977) *J. Assoc. Off. Anal. Chem.* **60**, 766–771

(4) Henderson, S. K., & McLean, L. A. (1979) *J. Assoc. Off. Anal. Chem.* **62**, 1358–1360

(5) Thompson, J. N., Hatina, G., & Maxwell, W. B. (1980) *J. Assoc. Off. Anal. Chem.* **63**, 894–898

PESTICIDE AND INDUSTRIAL CHEMICAL RESIDUES

Gas Chromatographic Determination of Picloram in Fish

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A simple method for determining picloram in fish is described. The sample is homogenized with ethyl acetate, acidified with 1N HCl, and extracted twice more with ethyl acetate. Ethyl acetate fractions are pooled, derivatized with diazomethane, cleaned up by column chromatography, and analyzed by electron capture gas chromatography. Rainbow trout exposed to ^{14}C-picloram were used to evaluate the efficiency of 2 methods of extraction and to provide data on the rate of uptake and the bioconcentration factor. The detection limit for this method is 5 ng/g, using a 4 g sample.

Picloram (4-amino-3,5,6-trichloropicolinic acid) is a widely used herbicide, effective in the control of many broadleaved weeds and woody plants (1). It degrades slowly in soils and water and is mobile in the soil environment because of its high water solubility (1). Thus, it has the potential to contaminate aquatic systems.

Methods reported in the literature for determining picloram and other acidic herbicides are time consuming and subject to low recoveries (2, 3). Questions have arisen concerning the severity of extraction conditions required to obtain satisfactory recoveries (3). Recoveries reported from experiments involving fortified biological substrates are not as conclusive as work done on substrates that were exposed to the chemical under field conditions. Absolute recovery efficiencies determined by using radiotracer techniques have not been reported so far.

This study describes and evaluates a method for determination of picloram in fish, by using HCl–ethyl acetate extraction, diazomethane derivatization, Florisil mini-column cleanup, and gas chromatographic (GC) determination.

METHOD

Apparatus

(a) *Gas chromatograph*—Model 5700A (Hewlett-Packard, Avondale, PA 19311) equipped with ^{63}Ni electron capture detector. Operating conditions: temperatures (°C)—injection port 250, column 195, detector 300; carrier gas argon–methane (95 + 5); flow rate 60 mL/min.

(b) *GC column.*—1.8 m × 3 mm glass, packed with 2% SE-30 + 3% QF-1 on 80–100 mesh Chromosorb W-HMDS.

(c) *Extraction apparatus.*—Polytron PT-10 (Brinkmann Instruments Inc., Westbury, NY 11590).

(d) *Sample combustion.*—Packard Model 306 oxidizer for determination of ^{14}C-content of fish by direct oxidation to ^{14}CO$_2$. Evolved CO$_2$ was collected in CO$_2$-M-Met (Amersham Corp., Arlington Heights, IL 60005) and diluted with PCS scintillation fluor (Amersham).

(e) *Liquid scintillation counting (LSC) apparatus.*—Beckman 7500. Quench corrections were made by use of an internal standard ratios method.

Reagents

Use pesticide grade solvents and reagent grade chemicals. Wash water with hexane before use.

(a) *Sodium sulfate.*—Anhydrous (Fisher Scientific Co.). Heat 12 h at 600°C and store in glass containers.

(b) *Diazomethane reagent in ethyl ether.*—Prepare from Diazald® (Aldrich Chemical Co., Milwaukee, WI 53233) according to instructions included with reagent. Diazomethane concentration is ca 12mg/mL.

(c) *Acidified water.*—Prepare by adding 20 mL concentrated HCl to 1 L hexane-washed, distilled water.

(d) *Picloram standard.*—99 + % pure (U.S. Environmental Protection Agency, Research Triangle Park, NC 27711).

(e) *^{14}C-Picloram.*—Uniformly ring-labeled, activity 1676 DPM/μg. Obtained from A. Smith, Agriculture Canada, Regina, Sask. Note: Standard (e) was 104% of Standard (d).

(f) *Florisil.*—(Floridin Co., Berkeley Springs, WV 25411). Prepare by heating 12 h at 450°C. Deactivate by adding 5 mL water to 95 g activated Florisil. Mix well by tumbling before use.

Fortification of Fish

Measured quantities of a solution of picloram in acetone were added to 4 g portions of ground whitefish in 25 × 150 mm test tubes, to final concentrations of 0.01, 0.025, 0.10, 0.25 μg/g (wet wt). Acetone was allowed to evaporate at room temperature and tubes were refrigerated until required for analysis.

Extraction

Two different extraction procedures were compared:

(1) Polytron extraction with acidification: Accurately weigh 4 g fish sample into 25 × 150 mm test tube. Add 20 mL ethyl acetate and homogenize with Polytron blender 30 s. Add 5 mL 1N HCl, vortex-mix, centrifuge, and transfer organic layer to round-bottom flask with Pasteur pipet. Repeat extraction of acidic residue with 2 more 20 mL portions of ethyl acetate, vortex-mixing and centrifuging each time. Pool all ethyl acetate extracts and concentrate on rotary evaporator to volume suitable for derivatization.

(2) Polytron extraction without acidification: Use same as procedure *(1)* but without addition of 1N HCl.

Derivatization

Take 1.0 mL aliquot of extract from extraction step, and place in 5 mL volumetric flask with 2 boiling granules and 0.1 mL methanol. Add 1 mL diazomethane solution. Let stand 1 h. Place in boiling water bath to decompose unreacted diazomethane and to reduce volume of solvent. Take just to dryness under gentle stream of nitrogen. Dissolve derivatized extract in ca 1 mL toluene for cleanup.

Note: Diazomethane is toxic, can cause specific sensitivity, and is potentially explosive. Prepare diazomethane reagent, methylate, and evaporate in fume hood, taking care to avoid contact with skin. Avoid ground glass joints, etched or scratched glassware, and sharp edges. Store diazomethane solutions in a freezer; do not expose to direct sunlight or strong artificial light.

Table 1. Recovery of ¹⁴C-activity from fish exposed to picloram (0.93 μg/mL), using ethyl acetate/Polytron with and without acidification with 1N HCl

Exposure time, h	Recovery,[a] %	
	With acidification	Without acidification
1	95.4	84.6
1	88.4	81.1
6	97.4	83.3
12	98.0	—
24	96.7	—
96	94.3	—
Mean % rec.[b]	95 ± 3.52	83 ± 1.77

[a] One analysis at each exposure time.
[b] Where % recovery = (activity (DPM) in extract × 100)/(total activity (DPM) extract + residue)

Table 2. Picloram concentration vs exposure time

Time, h	Picloram concn, μg/g wet[a]	Bioconcn factor (BCF)[b]
1	0.14	0.14
6	0.10	0.10
12	0.12	0.13
24	0.11	0.11
96	0.14	0.15

[a] Mean of 3 samples at each time for 1, 6, 12, and 24 h; mean of 2 samples for 96 h.
[b] BCF = activity DPM/g fish/activity DPM/mL water.

Table 3. Recovery of picloram added to whitefish tissue (and subjected to entire analytical procedure)

Fortification level, μg/g	Mean recovery,[a] %
0.01	103 ± 13%
0.025	98 ± 8%
0.10	98 ± 10%
0.25	98 ± 5%

[a] Mean of 3 analyses at each concentration ± SD.

Column Cleanup

Prepare mini-column by plugging Pasteur pipet with glass wool and adding 1.0 g 5% deactivated Florisil, tapping column gently to settle adsorbent. Prewash column with 10 mL toluene. Let solvent drain just to top of adsorbent. Quantitatively transfer to the column the concentrated, derivatized extract with three 1 mL rinsings of toluene. Elute column with additional 7 mL toluene. Discard this fraction. Elute column with 10 mL ethyl acetate. Ethyl acetate fraction contains methyl ester derivative of picloram. Concentrate on rotary evaporator and analyze by electron capture gas chromatography.

Recovery Studies

Exposure of Fish to ⁴C-Picloram

Rainbow trout (Salmo gairdneri) (1.5–4.5 g) were placed in a 20 L aquarium containing 15 L dechlorinated city tap water. After an adaptation period of 2 h, 4 control fish were removed and ¹⁴C-picloram was added to give a concentration of 0.93 μg/mL and an activity of 1118 DPM/mL (measured by LSC). Four fish each were removed at 1, 6, 12, 24, and 96 h post-treatment, carefully rinsed with water, and stored in glass vials at −20°C until required for analysis.

The extraction procedures were compared by extracting fish exposed to ¹⁴C-picloram as described, then measuring ¹⁴C-radioactivity in the extracts and residues.

Recoveries ranged from 88.4 to 96.7%.

Measurement of ¹⁴C-Radioactivity

Count extracts containing ¹⁴C by diluting with PCS–xylene (2 + 1) and counting by LSC. Combust aliquot of extracted fish (ca 200 mg air-dried material) on Packard 306 oxidizer. Collect ¹⁴CO$_2$ and count by LSC.

Results and Discussion

Table 1 presents a comparison of the efficiency of the 2 extraction procedures studied. The data were examined statistically using a difference of means test (4). The acidification step increased recovery from 83 to 95%, significant at $P = 0.01$. This is expected on the basis of the enhancement of picloram solubility in ethyl acetate at high acidity (1). The data also show that extraction efficiency remains high with fish exposed to picloram up to 96 h, which indicates that the combination of 1N HCl and ethyl acetate is sufficiently strong to release any picloram incorporated into the fish tissue by metabolic activity.

Table 2 presents data on the uptake of picloram as a function of exposure time. No significant increase in picloram concentration was seen during the time interval studied, which suggests that the picloram level in rainbow trout fry reaches a steady state in less than 1 h, at a picloram concentration of 1 ppm in water. The bioconcentration factor (BCF) data included in Table 2 (mean result 0.13) was calculated to give an estimate of the tendency of picloram to accumulate in rainbow trout fry. A BCF of 0.02 for picloram has been reported (1), but no details on the picloram concentration in the water, the exposure time, or the analysis procedure were given. The equilibrium BCF predicted from the water solubility of picloram using the equations of Kenaga (5) is 0.60.

Table 3 summarizes the recovery (98–103%) of different levels of picloram added to whitefish tissue and taken through the entire analytical procedure. The data indicate that the overall recovery remains constant, while the standard deviation is highest at the lowest concentration. This is partly because the lowest concentration level requires GC conditions that do not allow the recorder trace to return to baseline before the methyl ester of picloram elutes, which makes the peaks more difficult to measure accurately. The chromatograms shown in Figure 1 were obtained from whitefish extracts taken through the entire procedure. Pike and pickerel extracts were examined also and showed no interfering peaks. No other compounds were analyzed by this method, but the lack of extraneous peaks indicates the possibility that this method could be extended to acidic herbicides as a class.

Detection Limit

For the equivalent of 1.0 g fish derivatized, cleaned up, diluted to 2.0 mL, and injection of 2 μL, the GC conditions used resulted in a sensitivity of 1.0 ng/g per mm peak height. Using a minimum detectable peak height of 5 mm, the detection limit was 5 ng/g. Using the definition, minimum detectable peak height equals twice the recorder trace noise level, the detection limit becomes 1 ng/g. Figure 1 includes a chromatogram of a sample that was fortified at 0.01 μg/g to demonstrate these detection limits.

In conclusion, the analysis described was quick, simple, and sensitive. In addition, the radio-tracer study showed that the extraction step was efficient when dealing with fish actually exposed to picloram.
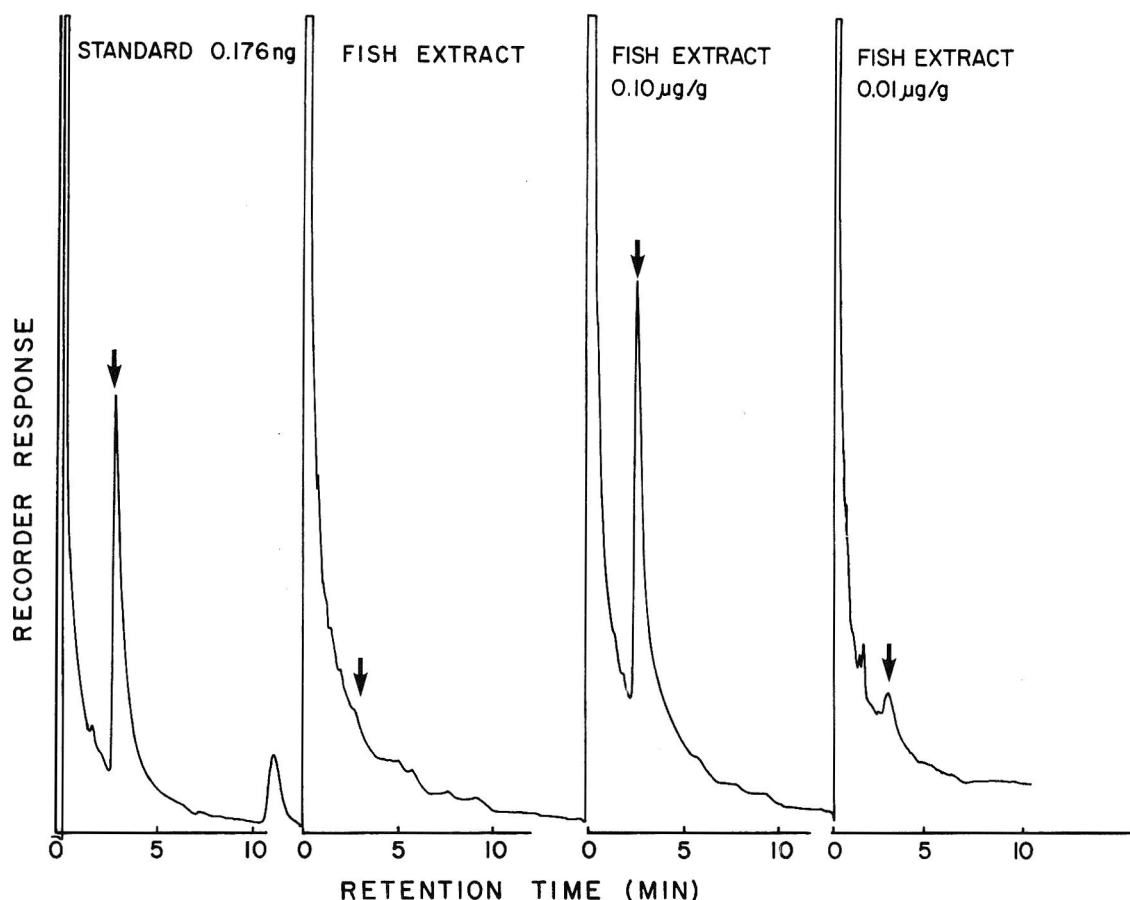
Figure 1. GC (EC detector) chromatograms of standard picloram solution, whitefish, and whitefish fortified with 0.10 μg/g of picloram, all subjected to entire analytical procedure.

REFERENCES

(1) *Picloram: The Effects of Its Use as a Herbicide on Environmental Quality* (1977) National Research Council of Canada Pub. No. NRCC 13684, NRCC, Ottawa, Ontario

(2) Moye, H. A. (Ed.) (1981) *Analysis of Pesticide Residues*, John Wiley & Sons, New York, NY, pp. 223–260

(3) *Phenoxy Herbicides—Their Effects on Environmental Quality* (1978) National Research Council of Canada Pub. No. NRCC 16075, NRCC, Ottawa, Ontario

(4) Steel, R. G. D., & Torrie, J. H. (1960) *Principles and Procedures of Statistics*, McGraw-Hill, New York, NY, pp. 73–74

(5) Kenaga, E. E. (1980) *Ecotoxicol. Environ. Safety* **4**, 26–38

Determination of Phosphine: Comparison of Rates of Desorption by Purge-and-Trap Method and by Sulfuric Acid Treatment

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Two methods were compared for quantitative determination of phosphine present on fumigated food and materials. The rate of desorption of PH₃ by using a purge-and-trap method was shown to be much slower when compared with sulfuric acid treatment and was also simpler. Application of the modified sulfuric acid treatment for real samples is described.

Phosphine has been widely used for years as a fumigant for disinfecting cereals and other stored food materials (1). The colorless gas is inflammable and highly toxic. It is produced in situ by the slow action of atmospheric and grain moisture on preparations that contain aluminum phosphide (AlP) and other ingredients that limit the rate of reaction and suppress combustion. Because the gas is toxic at low levels (2), it is necessary to monitor any residual gas in the fumigated materials.

Determination of phosphine has been the subject of many studies; chemical and colorimetric methods (3–11) have been used. Vinsjansen and Thrane (12), however, found that these methods lacked the desired sensitivity, specificity, and adaptability. On the other hand, gas chromatographic methods (GC) (13–17) were faster and more reliable at low concentrations of PH₃. Dumas (14) found that by using an N–P-specific detector, 0.005 mg PH₃/L could be detected. Other workers reported that the flame photometric detector was more sensitive and also gave rapid linear response and better reproducibility (15).

Dumas also (17) studied the purge-and-trap method for phosphine determination; amounts as low as 0.01 ng/sample could be determined quickly with reasonable accuracy. Sorption and desorption of PH₃ at different temperatures and as a function of time (18) were also studied by this author. Nowicki (19) investigated a different approach: The fumi-

gated wheat samples were refluxed with sulfuric acid, and the released phosphine was determined by GC with flame photometric detection.

In this paper, we present the results of a study of both the purge-and-trap method and sulfuric acid treatment; these methods were compared for their accuracy, reliability, and efficiency.

Experimental

Sampling

Samples, provided by the Ministry of Public Health, Kuwait, were fumigated with 20 g PH_3/ton and were aerated for various durations.

Preparation of Standards (19)

Weigh 3 mg Phostoxin tablet (Degesch, Frankfurt-on-Main, West Germany) in folded filter paper and place inside 1 L flask that has been purged with nitrogen. Phosphine is a highly toxic gas and makes an explosive mixture with air when present at 1.79% v/v. Therefore, air should be removed by purging with N_2, and phosphine should be contained in tightly stoppered, thick-walled flask and handled inside a fume hood.

Add 10 mL 10% H_2SO_4 to decompose aluminum phosphide. After 2 h, remove appropriate volume of headspace gas with gas-tight syringe and dilute in glass gas sampling vessel filled with nitrogen. Derive final concentration of standard by the fact that 3 mg pure Phostoxin yields 1 mg phosphine on complete decomposition.

Sulfuric Acid Treatment

The sulfuric acid treatment method for liberation of phosphine was simplified: Transfer 10–20 g sample to thick-walled flask filled with nitrogen. Add 10 mL 10% H_2SO_4 and tightly close flask with a rubber stopper containing septum. Heat flask to 60°C for ca 2 h. Immerse flask in cold water to condense water vapors, observing previously mentioned safety precautions. For analysis, use gas-tight syringe to remove sample from headspace gas through septum and inject directly into gas chromatograph.

Purge-and-Trap System

The purge and trap system described by Dumas (17) was modified (Figure 1): Sampling vessel, with screw caps, is connected to gas flow from injection port via stainless steel capillary tubing. Volume of sampling vessel is ca 450 mL. Sample vessel outlet is connected to column via stainless steel capillary tubing. Column, when used as a trap, is cooled in dry ice contained in styrofoam box. Effluent end of column is connected to flame photometric detector.

Place sample (20–50 g) inside vessel and purge nitrogen through it at 40 mL/min for appropriate length of time (0.5–1 h). After purging, analyze trapped phosphine by gas chromatography after removing dry ice and resuming normal chromatographic process.

Determination

Quantitate compound by using external standard method. Check detector response for linearity in the range 50 pg–10 ng, using for samples external standards of similar concentration range. Quantitative results showed good reproducibility and accuracy (Table 1).

Use gas chromatograph equipped with flame photometric detector and automated data calculation and integration. Optimize detector response by adjusting flow of flame gases. Under these conditions 30 pg phosphine produced reasonable
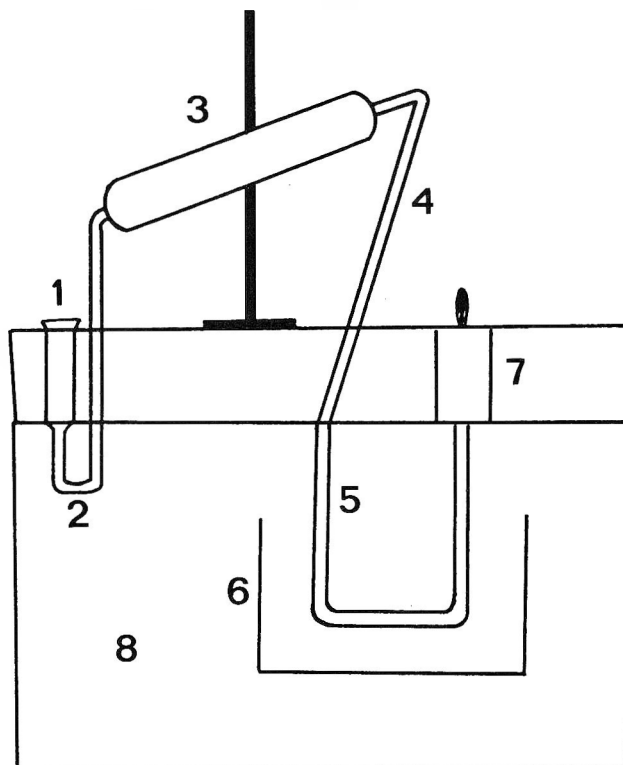
Figure 1. Purge-and-trap system for phosphine: (1) injection port; (2) connecting tube; (3) sampling vessel; (4) connecting tube; (5) column; (6) dry ice box; (7) FPD; (8) GC oven.

Table 1. Reproducibility and accuracy of PH_3 determinations, using external standard method

Amt injected, pg	No. of detns	SD(%)
50	3	1.15
100	5	3.13
200	3	3.96

response (S/N = 17). Although detection limits of 20 pg (20) and 5 pg (15) have been mentioned in the literature, no attempts were made to match these values.

Results and Discussion

Purge and On-Column Trap System

The purge-and-trap system suggested by Dumas (17) used two, 3-way switching valves that are placed inside the GC oven and therefore should be high temperature-tolerant. PH_3 from the samples was trapped on short traps filled with Chromosorb 102. A 30 min purge time was considered enough at a 40 mL N_2/min purge rate. The modification we introduced into the system eliminates the switching valves. Moreover, the column itself was used as the trap. Under the given conditions, the retention time of PH_3 on Chromosorb 102 at dry ice temperature and 40 mL N_2/min flow rate was more than 90 min.

Figure 2 shows the recovery of PH_3 after purging 30 min. Recovery efficiency of the system was quite satisfactory (90–95%), and we found that for an empty sampling vessel (approx. vol. 450 mL), a 30 min purge time was enough to remove all PH_3. The efficient recovery also reflected on the trapping, and it indicated the absence of any active absorbing sites for PH_3 in the system.
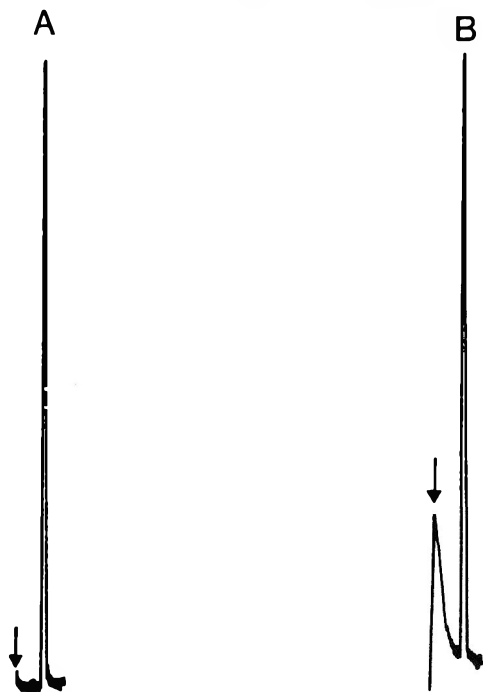
Figure 2. Gas chromatograms showing recovery efficiency of purge-and-trap system for PH₃: A, direct injection of 100 pg PH₃; B, purge-and-trap analysis of 100 pg PH₃; see *Experimental* for chromatographic conditions.

Table 2. Comparison of results obtained by purge-and-trap method and H₂SO₄ treatment (ng/kg)[a]

Sample	Aeration time, h	Purge-and-trap	H₂SO₄ treatment
Apricots	2	ND[b]	1660
Figs (loose)	2	130	200
Beans	2	740	101 000
Walnuts	2	7000	45 800
Apricot pulp	48	12	36 000
Dates	30[c]	ND	100

[a]Average of 5 determinations for each sample.
[b]ND = not detected (less than 1 ng/kg).
[c]Days.

Figure 3. Chromatograms showing efficiency of purging of fumigated bean sample: A, PH₃ collected after 1 h purge; B, after additional 1 h; C, additional 1 h; D, additional 1.5 h; see *Experimental* for chromatographic conditions.

Analysis of Fumigated Samples

After we established the recovery and overall efficiency of the system, actual samples were analyzed. Weighed samples were placed inside the sampling vessel and were purged 1 h to ensure complete removal of PH₃, which was then analyzed and quantitated.

To check for complete removal of PH₃, samples were purged and analyzed twice more (Figure 3). The first hour of purging did not remove all PH₃; in fact, the second hour of purging released more PH₃ than the first. Purging was continued and still more PH₃ was released. These results are completely contrary to the findings of Dumas (17), where 30 min of purging was considered enough to remove all PH₃. In our experience, some PH₃ was still left and could be recovered after purging 6 h. Similar results were obtained for other samples.

Sulfuric Acid Treatment

The other approach studied for PH₃ analysis was that suggested by Nowicki (19). The original system was simplified to enable us to prepare a larger number of samples simultaneously. PH₃, released as a result of H₂SO₄ treatment, was left in the flask instead of being transferred to another evacuated flask. Instead of boiling the sample 30 min with H₂SO₄,

we heated it to 60°C for about 2 h. Excessive pressure was reduced by condensing water vapors in the flask with cold water. This modified approach was used to analyze the samples by the purge-and-trap method. Table 2 lists the results of these analyses and those obtained by the purge-and-trap method. For purge-and-trap analysis, an average 1 h purge was used. The table clearly shows the significantly different results obtained by these 2 techniques. In some cases, the difference was 2 orders of magnitude and more.

Table 3 shows the comparison of rate of desorption of PH₃ from the sample. For the purge-and-trap method, the rate of desorption increases with increasing time. The maximum is not reached even after purging 4 h. In the sulfuric acid treatment, on the other hand, the maximum is reached after approximately 1.5 h and then remains steady.

One possible explanation for high values of PH₃ obtained by H₂SO₄ treatment is the presence of AlP dust on the sample, which is decomposed by H₂SO₄ to give PH₃. In the purge-and-trap method, AlP dust does not decompose during purging with dry N₂; however, from our experience, purging of PH₃ is not complete in a reasonable time for the given concentration ranges. Thus, for determination of PH₃ adsorbed on fumigated materials, the sulfuric acid treatment appears to be faster and simpler than the purge-and-trap method, under the conditions described above.

Table 3. Comparison of amount of PH$_3$ desorbed (ng/kg) as a function of time

Time, h	Purge-and-trap	H$_2$SO$_4$ method
0.5	210	48 000
1.0	740	78 000
1.5	1605	101 000
2.0	21 560	99 927
3.0	35 627	98 203
4.0	64 983	98 294

Acknowledgments

REFERENCES

(1) Chakrabarti, B., & Wainman, E. (1972) *Chem. Ind.* **72**, 300–302
(2) Christensen, H. E., & Luginbyhl, T. T. (Eds) (1974) U.S. Dept of Health and Human Services, Rockville, MD, pp. 599
(3) Feuersenger, M.(1955) *Dtsch. Lebensm. Rundsch.* **51**, 293–296
(4) Strache, F. (1960) *Dtsch. Lebensm. Rundsch.* **56**, 173–175
(5) Kroller, E. (1968) *Dtsch. Lebensm. Rundsch.* **64**, 6–9
(6) Romminger, K., & Kubel, D. (1971) *Ermahrungsforschung* **16**, 595–603
(7) British Standard Institution, BS 642 (1965) Appendix E, 20 pp.
(8) Dechant, R., Sanders, G., & Graul, R. (1966) *Am. Ind. Hyg. Assoc. J.* **27**, 75–77
(9) Muthu, M., Majummder, S. K., & Parpia, H.A.B. (1973) *J. Agric. Food Chem.* **21**, 184–186
(10) Hartkamp, H. (1974) Heft 32, Verlag W. Girardet, Essen, p. 37
(11) Kashi, K. P., & Muthu, M. (1975) *Pestic Sci.* **6**, 511–514
(12) Vinsjansen, A., & Thrane, K. E. (1978) *Analyst* **103**, 1195–1198
(13) Dumas, T. (1964) *J. Agric. Food Chem.* **12**, 257–260
(14) Dumas, T. (1969) *J. Agric. Food Chem.* **17**, 1164–1165
(15) Berck, B., Westlake, W. E., & Gunther, F. A. (1970) *J. Agric. Food Chem.* **18**, 143–147
(16) Berck, B., & Gunther, F. A. (1970) *J. Agric. Food Chem.* **18**, 148–153
(17) Dumas, T. (1978) *J. Assoc. Off. Anal. Chem.* **61**, 5–7
(18) Dumas, T. (1980) *J. Agric. Food Chem.* **28**, 337–339
(19) Nowicki, T. W. (1978) *J. Assoc. Off. Anal. Chem.* **61**, 829–836
(20) Robinson, W. H., & Hilton, H. W. (1971) *J. Agric. Food Chem.* **12**, 875–878

The Luke et al. Method for Determining Multipesticide Residues in Fruits and Vegetables: Collaborative Study

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Ten laboratories analyzed unfortified and fortified samples of lettuce, tomatoes, and strawberries for organochlorine and organophosphorus pesticides by applicable portions of the comprehensive multipesticide method of Luke et al. The 3 crops were fortified with 6 pesticides, α-BHC, dieldrin, chlorpyrifos, acephate, omethoate, and monocrotophos, each at 3 levels per crop. Included in the 54 fortifications were 16 pairs of blind duplicates: same pesticide, crop, and level. Recoveries were calculated by area comparisons with known reference materials, using the responses obtained from 2 separate element-specific gas chromatographic (GC) systems. The organochlorine pesticides were chromatographed on a methyl silicone column and detected with a Hall 700A electrolytic conductivity detector, and the organophosphorus pesticides were determined with a flame photometric detector after being chromatographed on a specified DEGS column material. Chlorpyrifos was quantitated on both GC systems. Mean recoveries ranged from 82.6% for acephate fortified at 0.5000 ppm in strawberries to 118.1% for 0.0636 ppm fortification of chlorpyrifos in lettuce. Interlaboratory coefficients of variation ranged from 4.0% for 0.6360 ppm fortification of chlorpyrifos in tomatoes to 17.8% for the 0.0636 ppm chlorpyrifos level in lettuce. The procedure features essentially no cleanup before GC and proved comparable to existing multiresidue methods for pesticides of the class types studied, as evidenced by the intra- and interlaboratory measurements of precision and recoveries obtained. The method with the 2 GC systems has been adopted official first action.

In 1975, Luke et al. (1) proposed an approach to the multiresidue analysis of fresh fruits and vegetables which was designed to eliminate or minimize cleanup steps, common to multiresidue methods that use gas chromatography (GC) for the determinative step. The advantages of this approach are increased analytical efficiency by decreasing the time for sample workup and increased recoverability for practically all classes of organic pesticide chemicals. The limiting factor of this approach is the availability of, or compatibility of the sample extract to, the chromatographic system(s) used in the determinative step(s). This limitation prevented initial general acceptance of the approach for organochlorine residues because a Florisil cleanup step was essential before using the electron capture (EC) detector, which negated any real time saving from the established Mills et al. (2) procedure. In addition, residues of the more polar organophosphorus and some organonitrogen pesticides could be isolated by other multiresidue procedures (3, 4) that yielded a cleaner sample extract to chromatograph. The chromatography of these more polar pesticides, regardless of the methods used for their isolation, was difficult because a reliable column packing material was not available which routinely reproduced desirable chromatographic characteristics. The dirtier extracts obtained from the Luke et al. approach tended to magnify these chromatographic problems.

In 1981, Luke et al. (5) modified their method by substituting a halogen-selective Hall 700A electrolytic conductivity detector (HECD) for the EC detector, which allowed elimination of the Florisil cleanup step without sacrificing sensitivity. They also studied the chromatography of the polar compounds and compiled a retention list, relative to parathion, of over 80 organophosphorus compounds obtained with a commercially available DEGS column packing. Subsequent inter- and intralaboratory trials in Food and Drug Administration (FDA) laboratories demonstrated that reten-

tion values similar to those published could be reproduced within an approximate 10% range with different lots of the DEGS material obtained from the same commercial supplier. Both the retention data and the HECD modification have been incorporated in recent revisions of the *Pesticide Analytical Manual* (PAM) (6).

The comprehensive analytical approach proposed by Luke et al. (5) would involve up to 8 different column/detector combinations per sample, and ideally this would provide the capability to detect, quantitate, and confirm the presence of most organochlorine, -phosphorus, -sulfur, -nitrogen, and other miscellaneous pesticide types. This collaborative study addressed only the chlorine- and phosphorus-containing classes and used one separate column/detector combination for each class. The purposes of the study were *(1)* to compare the interlaboratory performance of the Luke et al. approach to the existing official multiresidue methods (7); and *(2)* to provide a base from which this comprehensive analytical approach can be expanded in the future.

Collaborative Study

The study required the collaborators to analyze a total of 12 samples consisting of 1 unfortified and 3 fortified portions each of lettuce, tomatoes, and strawberries by the Luke et al. (5) method as modified for inclusion in the PAM (6). A copy of the method along with performance criteria of the 2 required GC systems as they appear in the PAM were supplied. Individual 100 g samples prepared from commercially available fresh produce were supplied in screw-top, 4 oz glass jars. Samples were prepared by chopping ca 20 lb produce in a 40 qt cutter/mixer, followed by intermittent blending of the resultant slurry during weighing to minimize moisture separation. Fortified portions were prepared by pipetting 1 mL acetone solution, containing known concentrations of the 6 pesticide chemicals studied, directly into the weighed product. Unfortified portions were treated identically with 1 mL acetone only. The sample jars were then closed with foil-lined screw caps, and the samples were frozen.

Before starting the study, collaborators were required to order DEGS column packing material and pesticide reference standards from specified sources. The column material (2% stabilized DEGS on 80–100 mesh Chromosorb W AW) source was Analabs Inc., 80 Republic Dr, North Haven, CT 06473. The source for the reference standards was the Environmental Protection Agency through Northrup Services Inc., PO Box 12313, Research Triangle Park, NC 27709, and included α-BHC, dieldrin, chlorpyrifos, acephate, omethoate, and monocrotophos.

The chromatographic system required for the organochlorine determinations consisted of a methyl silicone (e.g., OV-101) column and a Hall 700A EC detector operated in the halogen-selective mode. Both systems were to be operated at conditions that would closely duplicate the retention data published in Table 331-A of the PAM (6). For the organophosphorus determinations, a 4 ft × 2 mm glass column packed with the special order 2% DEGS material was specified as was the use of a phosphorus-selective flame photometric detector (FPD). Both systems were to be operated at a sensitivity that would produce ca 50% recorder response for 1.5 ng chlorpyrifos. In addition, the FPD system was expected to produce ca 50% recorder response for 6 ng monocrotophos. Each system was to be monitored at least twice daily with a mixed standard to check column/detector performance. Compounds suggested for checking chromatographic performance were chlorpyrifos, dieldrin, and *p,p'*-DDT for the methyl silicone/HECD system and methamido-

phos, chlorpyrifos, and monocrotophos for the DEGS/FPD system. Collaborators were expected to take corrective action if performance deteriorated.

All standard solutions, those used for performance checks as well as those used for quantitation, were to be prepared in acetone suitable for pesticide residue determination and were to be refrigerated when lengthy storage periods (including overnight) were anticipated. Equilibration of the solution for ca 1 h at room temperature was specified before use for quantitation. The concentration of each individual working standard was to be prepared such that a 3–4 μL injection would produce a 30–70% recorder response on properly functioning GC systems.

Before proceeding with the recovery determinations, the collaborators were instructed to concurrently analyze the unfortified portions from each commodity. This step was to allow familiarization with the method, to ensure that the instructions and the requirements of the study were clearly stated, and to check the performance of the chromatographic systems with sample extracts. Initial sample injections were to be equivalent to ca 12 mg sample weight and all identifiable residues were to be quantitated. All unidentifiable chromatographic responses obtained, using the 2 specified GC systems, were to be reported by retention time relative to chlorpyrifos. After satisfactory completion of this step, collaborators were instructed to continue the study by concurrently analyzing the 3 fortified portions of a single commodity. All analytical steps, including calculations, were to be completed before proceeding with the next commodity. It was specified that chlorpyrifos be determined on both GC systems.

All analyses were initiated by quantitatively transferring each 100 g sample portion to a blender jar with the aid of 200 mL acetone extracting solvent. All final sample dilutions were measured accurately in either volumetric flasks or graduated conical tubes that were calibrated with acetone delivered from a buret. In the latter case, the calibrated volumes had to be used to obtain the sample weight/volume relationship. Collaborators were required to use peak area comparison for quantitation. Areas of the fortified residue and its corresponding reference standard were to be within ±25% of each other. The use of electronic integration was encouraged for expediency, but any method of area determination could be used. Collaborators were informed that all area measurements would be verified by the manual method proposed by Condal-Bosch (8). To facilitate obtaining the accurate manual measurements that this procedure requires, it was specified that the chromatographic peaks be broadened by using increased recorder/integrator scan speeds. Minima of 1/2 in./min were specified for strip chart recorders and 1 cm/min for electronic integrators.

Organochlorine and Organophosphorus Pesticide Residues
Gas Chromatographic Method
First Action

(Applicable to nonfatty, high moisture foods
(<2 g fat/100 g).)

Principle

Nonfatty sample is blended with acetone and filtered; pesticides are transferred from aq. filtrate to org. phase by shaking with pet. ether and CH_2Cl_2; after drying, org. phase is concd in presence of pet. ether and then acetone to remove CH_2Cl_2; aliquot of concd org. phase is injected into various GC systems for detn of wide variety of pesticide residues.

Absence of cleanup steps permits examination for residues of many chem. types, including many that would not be

recovered thru methods requiring Florisil or charcoal column chromatgc step.

Reagents and Apparatus

(a) *Solvents.*—Acetone, CH_2Cl_2, pet. ether, and ethyl ether, distd in glass, or equiv. (Burdick & Jackson Laboratories).

(b) *Sodium sulfate.*—Anhyd., granular.

(c) *Glass wool.*—Rinse with acetone and alcohol several times and dry. Washed glass wool will be somewhat brittle.

(d) *High-speed blender.*—Waring Blendor, or equiv.

(e) *Chromatographic tubes.*—With Teflon stopcocks, 22 mm × 300 or 400 mm long.

(f) *Kuderna-Danish concentrator.*—500 mL with Snyder column and fitted with vol. flask or graduated receiving tube. Calibrate receiving tube with acetone delivered from a buret. Use buret corrected volume for sample wt calcn.

(g) *Separatory funnels.*—1 L, with Teflon stopcocks.

(h) *Gas chromatograph.*—*(1) For organochlorine residues.*—Instrument contg any suitable methyl silicone column, such as 2% OV-101, on 80–100 mesh Chromosorb W (HP), 6 ft × 2 mm id glass, and Hall 700A HECD halogen-specific detector. Column, 200°; He carrier gas, 60 mL/min; detector 900°, H reaction gas 60–100 mL/min; *n*-propanol solv. 0.35 mL/min; electrometer range 10 in OPR/FLT mode; attenuation 5. *(2) For organophosphorus residues.*—Instrument with column contg 2% stabilized DEGS on 80–100 mesh Chromosorb W (HP), 4 ft × 2 mm id silanized glass, and P-specific flame photometric detector (526 nm filter). Column 180°; detector 200°; He carrier gas, 60 mL/min. Condition column (disconnect detector) by passing carrier gas thru column 0.5 h at ≤80°. Program temp. at 1–2°/min to 230° and hold overnight. Establish stable flame at electrometer setting that will produce 50% full scale deflection for 1.5 ng chlorpyrifos and 6 ng monocrotofos. If necessary, increase air/O until ≥50% response. Baseline noise should be <2%.

(i) *Reference std materials.*—BHC, dieldrin, chlorpyrifos, *p,p'*-DDT, acephate, omethoate, methamidophos, and monocrotophos (U.S. Environmental Protection Agency, Pesticides and Industrial Chemical Repository, Las Vegas, NV 89114). Prep. all stds in acetone. *Mixed stds.*—For Hall system, std soln should contain at least chlorpyrifos, dieldrin, and *p,p'*-DDT. For flame photometric detector, std soln should contain at least methamidophos and chlorpyrifos. Do not use mixed std solns for quant. of unknowns.

(j) *Std solns.*—Prep. all stock solns and dilns in glass-distd acetone. Prep. GC std solns so 4 μL injection causes 30–70% full scale deflection in properly functioning system. Suggested concns are given below. Check responses before beginning analysis of blanks, spikes, or samples. Store all std solns in tightly stoppered containers in refrigerator. Let equilibrate 1 h at room temp. before using.

Compd	ng/μL
α-BHC	0.1
chlorpyrifos	0.5
dieldrin	0.2
acephate	0.5
omethoate	2.0
monocrotophos	2.0
methamidophos	0.2
p,p'-DDT	0.5

Preparation of Sample

Chop or blend fruits and vegetables and mix thoroly. Weigh 100 g chopped or blended sample into high-speed blender jar,

add 200 mL acetone, and blend 2 min at high speed. *Do not* add Celite. Filter with suction thru 12 cm Buchner funnel fitted with sharkskin paper. (Note: Rinse filter paper with acetone before filtration of sample to remove artifacts that can interfere with analysis.) Collect ext in 500 mL suction flask. Filtration is normally complete in <1 min. Continuation of vac. for excessive period can reduce vol. of ext and cause error in calcn.

Place 80 mL sample ext in 1 L sep. funnel, and add 100 mL pet. ether and 100 mL CH_2Cl_2. Shake vigorously 1 min. Transfer lower aq. layer to second 1 L sep. funnel. Dry upper org. layer in first sep. funnel by passing thru ca 1½ in. Na_2SO_4 supported on washed glass wool in 4 in. funnel, collecting in 500 mL Kuderna-Danish concentrator fitted with vol. flask or calibrated receiving tube. To sep. funnel with aq. phase, add 7 g NaCl and shake vigorously 30 s until most NaCl is dissolved. Add 100 mL CH_2Cl_2, shake 1 min, and dry lower org. phase thru same Na_2SO_4. Ext aq. phase with addnl 100 mL CH_2Cl_2, and dry as above. Rinse Na_2SO_4 with ca 50 mL CH_2Cl_2. Attach Snyder column on Kuderna-Danish concentrator (boiling chips may be added) and start evapn slowly by placing only receiver tube into steam. After 100–150 mL has evapd, concentrator may be exposed to more steam. When liq. level in hot concentrator tube is ca 2 mL, add 100 mL pet. ether thru Snyder column and reconc. to ca 2 mL. Add 50 mL pet. ether and repeat concn step. Add 20 mL acetone and reconc. to ca 2 mL. Do not let soln go to dryness during any concn step. Adjust vol. of ext to suitable definite vol. with acetone.

Calculation of equivalent sample weight.—Calc. equiv. sample wt in final soln as follows:

$$\frac{\text{mg sample equiv.}}{\mu L \text{ final ext}} = 100 \times \frac{80}{200 + W - 10} \times \frac{1}{\text{mL final vol.}}$$

where 200 = mL acetone blended with 100 g sample; W = amt (mL) H_2O present in sample; and 10 = adjustment for water–acetone vol. contraction. Thus, when sample contains 85% H_2O (85 mL/100 g) and final ext vol. is 7 mL, each μL contains:

$$100 \times \frac{80}{200 + 85 - 10} \times \frac{1}{7}$$

$$= 4.15 \text{ mg sample equiv./}\mu L \text{ final ext}$$

Determination

Check that both GC systems are working properly by injecting mixed std soln into each. Inject ca 12 mg sample equiv. into each system. Tentatively identify any GC responses on basis of retention times. Quantitate residue peak(s) by area comparison with that obtained from known amt of ref. material(s). To ensure valid measurement of residue amt, area of peaks from residue and ref. std should be within ±2.5%. *Caution:* Repeated injection of sample exts which have had min. cleanup can be detrimental to GC columns. Replace packing material at front of GC columns as needed to maintain chromatgc quality and prolong column life.

Results and Discussion

The 3 commodities selected for the study included a leafy green vegetable (lettuce), a high acid fruiting vegetable (tomato), and a high sugar fruit (strawberry). Besides being high consumption items, they were chosen in part to demonstrate the applicability of the procedure with highly varied product matrices. The moisture contents of these commodities were experimentally determined to be 95.6, 95.3, and 91.2%, respectively, for lettuce, tomatoes, and strawberries.

Table 1.   Collaborative fortification levels (ppm) of 6 pesticides by commodity and sample number[a]

Commodity	No.	α-BHC	Dieldrin	Chlorpyrifos	Acephate	Omethoate	Monocrotophos
Lettuce	1	1.044	0.0844	0.0636	10.00	1.004	1.000
	2	1.044	0.1164	0.6360	10.00	2.008	1.000
	3	0.3096	0.0844	0.0636	1.000	2.008	0.3000
Tomatoes	1	0.5160	0.1688	0.6360	4.000	1.004	0.5000
	2	0.3096	0.1164	0.3816	4.000	2.008	0.3000
	3	1.044	0.1164	0.6360	1.000	1.004	0.5000
Strawberries	1	0.5160	0.0844	0.6360	0.5000	0.5020	0.3000
	2	1.044	0.1688	0.3816	1.000	2.008	0.3000
	3	1.044	0.1164	0.3816	0.5000	0.5020	0.5000

[a]Underlined values are blind duplicates.

Table 2.   Collaborative results (ppm) of 3 chlorinated pesticides recovered from lettuce (3 samples) by the Luke et al. method, using methyl silicone column GC with halogen-specific EC detection (Hall 700A)

Coll.	α-BHC 1	α-BHC 2	α-BHC 3	Dieldrin 1	Dieldrin 2	Dieldrin 3	Chlorpyrifos 1	Chlorpyrifos 2	Chlorpyrifos 3
1	0.901	0.788	0.257	0.071	0.099	0.082	0.070	0.620	0.072
2	1.22	1.16	0.377	0.079	0.134	0.093	0.075	0.701	0.066
3	1.11	1.20	0.383	0.081	0.109	0.084	0.068	0.568	0.072
4	(1.04)[a]	(1.83)	(0.478)	(0.117)[b]	0.133	0.112	0.089	0.699	0.080
5	1.05	1.11	0.307	0.079	0.112	0.077	0.074	0.652	0.066
6	0.989	1.04	0.279	0.076	0.107	0.076	0.068	0.649	0.054
7	1.06	1.07	0.312	0.089	0.114	0.118	0.071	0.663	0.103
8	1.02	1.07	0.367	0.084	0.116	0.090	0.070	0.769	0.084
9	0.983	0.988	0.303	0.081	0.112	0.085	0.070	0.706	0.070
10	1.22	1.15	0.361	0.094	0.138	0.108	0.077	0.884	0.084
Amt added	1.044	1.044	0.3096	0.0844	0.1164	0.0844	0.0636	0.6360	0.0636
Av. found	1.061	1.064	0.3273	0.0816	0.1174	0.0925	0.0732	0.6911	0.0751
Std dev.	0.1070	0.1224	0.0458	0.0068	0.0130	0.0150	0.0063	0.0869	0.0134
Mean %	101.6	101.9	105.6	96.7	100.9	109.6	115.1	108.7	118.1
CV, %	10.1	11.5	14.0	8.4	11.1	16.2	8.6	12.6	17.8

[a]Results in parentheses from Collaborator 4 (unless footnoted further) are excluded from statistical summary; standard degradation or preparation problem suspected.
[b]Excluded by Dixon test at $P > 95\%$.

Table 3.   Collaborative results (ppm) of 4 organophosphorus pesticides recovered from lettuce (3 samples) by the Luke et al. method, using DEGS column GC with phosphorus-selective FPD

Coll.	Chlorpyrifos 1	Chlorpyrifos 2	Chlorpyrifos 3	Acephate 1	Acephate 2	Acephate 3	Omethoate 1	Omethoate 2	Omethoate 3	Monocrotophos 1	Monocrotophos 2	Monocrotophos 3
1	0.068	0.607	0.067	8.90	9.17	0.869	1.04	2.11	2.14	1.11	1.18	0.391
2	0.076	0.760	0.080	9.13	9.80	1.04	1.22	2.10	2.01	1.28	1.22	0.380
3	0.069	0.669	0.070	10.3	9.94	1.02	1.11	2.22	2.16	1.11	1.07	0.353
4	0.085	0.787	0.076	(5.64)[a]	(6.58)	(0.665)	0.974	1.79	1.97	1.05	1.07	0.318
5	0.072	0.688	0.060	8.95	9.34	0.922	1.02	2.08	1.97	1.02	1.04	0.255
6	0.064	0.606	0.061	8.04	8.97	0.842	0.901	2.08	1.66	0.952	1.05	0.292
7	0.068	0.672	0.075	9.14	9.26	0.840	1.07	2.23	2.02	0.967	1.08	0.298
8	0.076	0.757	0.076	8.93	9.41	0.913	1.22	2.18	2.33	1.12	1.11	0.331
9	0.074	0.648	0.055	8.49	10.3	0.922	1.22	2.56	2.48	1.09	1.13	0.323
10	0.085	0.807	0.080	9.01	7.68	0.963	1.20	2.15	2.35	1.09	1.07	0.336
Amt added	0.0636	0.6360	0.0636	10.00	10.00	1.000	1.004	2.008	2.008	1.000	1.000	0.3000
Av. found	0.0737	0.7001	0.0700	8.99	9.32	0.926	1.098	2.150	2.109	1.079	1.102	0.3277
Std dev.	0.0071	0.0730	0.0089	0.605	0.744	0.0717	0.1153	0.1897	0.2371	0.093	0.059	0.0408
Mean %	115.9	110.1	110.1	89.9	93.2	92.6	109.4	107.1	105.0	107.9	110.2	109.2
CV, %	9.6	10.4	12.7	6.7	8.0	7.7	10.5	8.8	11.2	8.6	5.3	12.5

[a]See Table 2.

These values compared well with published values (9) of 95.5, 93.5, and 89.9%. Collaborators were instructed to use a 95% moisture factor for lettuce and tomatoes and a 90% factor for strawberries to determine sample weight equivalents.

Table 1 summarizes the amounts of each pesticide added to the sample portions of each commodity. Within the 54 fortifications, there were 16 pairs of blind duplicates: same pesticide level on the same commodity in a different sample. With the dual quantitation requirement for chlorpyrifos, each collaborator had to report 19 blind duplicate values. In addition, 12 of the 16 blind duplicate levels were repeated using at least 1 other commodity. Each pesticide was fortified at 3 different levels with the exception of acephate, which had 4 levels of fortification. In cases where a tolerance was known to exist for a particular pesticide/commodity combination (10), fortification at that tolerance value was closely approximated and was determined in duplicate.

Tables 2–7 present individual collaborative results by commodity and detection system. Eight collaborators used area comparisons for quantitation as instructed. The results from Collaborators 1, 4, 7, and 8 were calculated from areas obtained with electronic integration. Collaborators 3, 5, 6, and 9 used

Table 4. Collaborative results (ppm) of 3 chlorinated pesticides recovered from tomatoes (3 samples) by the Luke et al. method, using methyl silicone GC with halogen-specific EC detection (Hall 700A)

Coll.	α-BHC			Dieldrin			Chlorpyrifos		
	1	2	3	1	2	3	1	2	3
1	0.511	0.272	0.941	0.162	0.116	0.098	0.714	0.399	0.607
2	0.621	0.329	1.29	0.158	0.106	0.117	0.616	0.391	0.608
3	0.601	0.261	1.17	0.196	0.126	0.130	0.717	0.339	0.681
4	(0.744)[a]	(0.447)	(1.95)	0.229	0.122	0.119	0.660	0.408	0.683
5	0.499	0.287	1.04	0.162	0.106	0.107	0.599	0.372	0.669
6	0.469	0.255	0.988	0.153	0.085	0.087	0.588	0.362	0.588
7	0.447	0.297	1.07	0.172	0.106	0.115	0.651	0.384	0.635
8	0.507	0.322	1.00	0.170	0.130	0.082	0.656	0.384	0.648
9	0.504	0.300	1.02	0.178	0.126	0.126	0.666	0.392	0.631
10	0.588	0.345	1.12	0.174	0.149	0.131	0.706	0.391	0.653
Amt added	0.5160	0.3096	1.044	0.1688	0.1164	0.1164	0.6360	0.3816	0.6360
Av. found	0.5186	0.2964	1.071	0.1754	0.1172	0.1112	0.6573	0.3822	0.6403
Std dev.	0.0629	0.0311	0.1076	0.0224	0.0175	0.0173	0.0462	0.0200	0.0325
Mean %	100.5	95.7	102.6	103.9	100.7	95.5	103.3	100.2	100.7
CV, %	12.1	10.5	10.0	12.8	15.0	15.6	7.0	5.2	5.1

[a]See Table 2.

Table 5. Collaborative results (ppm) of 4 organophosphorus pesticides recovered from tomatoes (3 samples) by the Luke et al. method, using DEGS column GC with phosphorus-selective FPD

Coll.	Chlorpyrifos			Acephate			Omethoate			Monocrotophos		
	1	2	3	1	2	3	1	2	3	1	2	3
1	0.654	0.419	0.583	3.73	3.72	0.942	1.22	2.32	1.08	0.621	0.343	0.494
2	0.670	0.406	0.688	4.11	3.27	0.949	1.20	2.18	1.03	0.628	0.347	0.560
3	0.716	0.407	0.690	3.84	3.38	0.918	1.09	2.08	1.04	0.576	0.327	0.538
4	0.717	0.419	0.720	(2.77)[a]	(2.40)	(0.626)	(1.58)[b]	1.75	0.901	0.566	0.349	0.573
5	0.682	0.348	0.639	3.64	3.83	0.892	1.09	1.93	1.09	0.512	0.306	0.530
6	0.647	0.353	0.564	3.17	3.45	0.780	1.04	1.90	0.860	0.512	0.306	0.436
7	0.660	0.391	0.675	3.51	3.67	0.916	1.12	2.25	1.05	0.506	0.326	0.506
8	0.693	0.405	0.727	3.29	3.39	1.10	1.01	1.91	1.10	0.497	0.270	0.496
9	0.714	0.418	0.664	3.43	3.56	1.01	1.20	2.32	1.20	0.486	0.357	0.581
10	0.658	0.472	0.794	3.24	3.05	0.793	1.14	2.10	1.26	0.532	0.329	0.525
Amt added	0.6360	0.3816	0.6360	4.000	4.000	1.000	1.004	2.008	1.004	0.5000	0.3000	0.5000
Av. found	0.6811	0.4038	0.6744	3.551	3.480	0.9222	1.123	2.074	1.061	0.5436	0.3260	0.5239
Std dev.	0.0274	0.0353	0.0679	0.3087	0.2425	0.0989	0.0737	0.1963	0.1198	0.0513	0.0262	0.0434
Mean %	107.1	105.8	106.0	88.8	87.0	92.2	111.9	103.3	105.7	108.7	108.7	104.8
CV, %	4.0	8.7	10.1	8.7	7.0	10.7	6.6	9.5	11.3	9.4	8.0	8.3

[a,b]See Table 2.

a combination of electronic integration and the manual Condal-Bosch (8) method and usually reported both values. When the collaborator specified a value considered to be "more accurate," only this value was tabulated. When values obtained by the 2 methods of quantitation were reported and neither value was preferentially specified, the average of the 2 values was tabulated. Collaborator 10 used the manual measurement technique exclusively, and for the most part, the results would have been identical to peak height calculations because extremely symmetrical peak shapes were obtained. Collaborator 2 reported values based only on peak height comparisons, and unlike Collaborator 10, significant peak tailing occurred on both chromatographic systems. Peak tailing did affect the quantitation because chromatographic peaks from the reference standards in the neat acetone solvent tended (not exclusively) to tail more than peaks from the standards recovered in the final sample solution that contained product coextractives. Recalculation of these results using the manual Condal-Bosch (8) measurements and comparison with the reported peak height values showed that 13 of the 63 results differed by > 10%. To minimize the bias introduced by the tailing peaks and that possibly introduced during the calculation of "known" values, the tabulated results for Collaborator 2 represent an average of both peak height and Condal-Bosch (8) quantitation methods. The peak tailing problem had been previously noted in a preliminary method trial

involving 3 laboratories and was the basis for requiring area comparisons for quantitation in this study.

The results for the 63 individual sets of data presented in Tables 2–7 range from a low average recovery of 82.6% for a 0.5000 ppm level of acephate in strawberries to a high average recovery of 118.1% for a 0.0636 ppm level of chlorpyrifos (by HECD) in lettuce. Coefficients of variation (CVs) for the 63 sets of data ranged from 4.0% for a 0.6360 ppm level of chlorpyrifos in tomatoes (FPD) to 17.8% for the 0.0636 ppm chlorpyrifos in lettuce discussed above. A duplicate sample of chlorpyrifos in lettuce at the 0.0636 ppm level averaged 115.1% recovery with a CV of only 8.6%.

A summary of the collaborative results that appear in Tables 2–7 for the ranges of average recoveries and CVs for the individual pesticides is as follows: (1) α-BHC average recovery ranged from 95.7% (0.3096 ppm in tomatoes) to 105.6% (0.3096 ppm in lettuce) and CVs ranged from 9.6% (1.044 ppm in strawberries) to 15.2% (0.5160 ppm in strawberries); (2) dieldrin average recovery ranged from 95.5% (0.1164 ppm in tomatoes) to 110.0% (0.0844 ppm in strawberries) and CVs ranged from 8.4% (0.0844 ppm in lettuce) to 16.2% (0.0844 ppm in lettuce); (3) chlorpyrifos (HECD) average recovery ranged from 100.2% (0.3816 ppm in tomatoes) to 118.1% (0.0636 ppm in lettuce) and CVs ranged from 5.1% (0.6360 ppm in tomatoes) to 17.8% (0.636 ppm in lettuce); (4) chlorpyrifos (FPD) average recovery ranged from 102.5% (0.3816

Table 6. Collaborative results (ppm) of 3 chlorinated pesticides recovered from strawberries (3 samples) by the Luke et al. method, using methyl silicone GC with halogen-specific EC detection (Hall 700A)

Coll.	α-BHC			Dieldrin			Chlorpyrifos		
	1	2	3	1	2	3	1	2	3
1	0.396	0.786	1.09	0.073	0.153	0.108	0.546	0.400	0.427
2	0.563	1.20	1.14	0.098	0.157	0.131	0.642	0.483	0.489
3	0.562	1.14	1.00	0.094	0.195	0.129	0.834	0.484	0.469
4	(1.34)[a]	(2.59)	(2.69)	0.112	0.226	0.114	0.623	0.400	0.428
5	0.427	1.05	0.913	0.080	0.170	0.114	0.616	0.403	0.388
6	0.444	0.930	0.923	0.085	0.132	0.101	0.626	0.345	0.332
7	0.487	1.07	0.991	0.091	0.198	0.131	0.736	0.494	0.491
8	0.497	0.865	0.934	0.086	0.182	0.116	0.673	0.468	0.369
9	0.492	1.01	1.05	0.102	0.155	0.134	0.682	0.447	0.422
10	0.637	1.19	1.19	0.107	0.202	0.133	0.741	0.544	0.416
Amt added	0.5160	1.044	1.044	0.0844	0.1688	0.1164	0.6360	0.3816	0.3816
Av. found	0.5001	1.027	1.026	0.0928	0.1770	0.1211	0.6719	0.4468	0.4231
Std dev.	0.0759	0.1439	0.0989	0.0122	0.0286	0.0119	0.0814	0.0591	0.0512
Mean %	96.9	98.4	98.3	110.0	104.9	104.0	105.6	117.1	110.9
CV, %	15.2	14.0	9.6	13.2	16.1	9.8	12.1	13.2	12.1

[a] See Table 2.

Table 7. Collaborative results (ppm) of 4 organophosphorus pesticides recovered from strawberries (3 samples) by the Luke et al. method, using DEGS column GC with phosphorus-selective FPD

Coll.	Chlorpyrifos			Acephate			Omethoate			Monocrotophos		
	1	2	3	1	2	3	1	2	3	1	2	3
1	(0.472)[b]	0.346	0.386	0.312	0.694	0.375	0.395	1.61	0.502	0.294	0.269	0.463
2	0.676	0.447	0.417	0.376	0.757	0.378	0.479	2.16	0.489	0.379	0.320	0.503
3	0.684	0.393	0.400	0.523	0.900	0.434	0.532	2.07	0.497	0.340	0.322	0.529
4	0.681	0.457	0.428	(0.283)[a]	(0.620)	(0.279)	0.463	2.16	0.465	0.347	0.354	0.600
5	0.601	0.368	0.348	0.434	0.833	0.448	0.536	1.96	0.553	0.319	0.315	0.568
6	0.596	0.362	0.365	0.375	0.872	0.404	0.447	2.12	0.455	0.275	0.294	0.459
7	0.694	0.432	0.422	0.443	0.877	0.454	0.521	2.28	0.566	0.318	0.348	0.514
8	0.654	0.417	0.332	0.455	0.883	0.458	0.576	2.20	0.530	0.332	0.261	0.490
9	0.663	0.413	0.407	0.386	0.879	0.395	0.614	2.49	(0.702)[b]	0.343	0.357	0.542
10	0.696	0.478	0.407	0.419	0.796	0.370	0.520	1.98	0.462	0.302	0.297	0.484
Amt added	0.6360	0.3816	0.3816	0.5000	1.000	0.5000	0.5020	2.008	0.5020	0.3000	0.3000	0.5000
Av. found	0.6606	0.4113	0.3912	0.4137	0.8323	0.4129	0.5083	2.103	0.5021	0.3249	0.3137	0.5152
Std dev.	0.0377	0.0437	0.0327	0.0602	0.0698	0.0359	0.0640	0.2302	0.0401	0.0299	0.0337	0.0454
Mean %	103.9	107.8	102.5	82.7	83.2	82.6	101.3	104.7	100.0	108.3	104.6	103.0
CV, %	5.7	10.6	8.4	14.5	8.4	8.6	12.6	10.9	8.0	8.7	10.7	8.8

[a,b] See Table 2.

ppm in strawberries) to 115.9% (0.0636 ppm in lettuce) and CVs ranged from 4.0% (0.6360 ppm in tomatoes) to 12.7% (0.0636 ppm in lettuce); (5) acephate average recovery ranged from 82.6% (0.5000 ppm in strawberries) to 93.2% (10.00 ppm in lettuce) and CVs ranged from 6.7% (10.00 ppm in lettuce) to 14.5% (0.5000 ppm in strawberries); (6) omethoate average recovery ranged from 100.0% (0.5020 ppm in strawberries) to 111.9% (1.004 ppm in tomatoes) and CVs ranged from 6.6% (1.004 ppm in tomatoes) to 12.6% (0.5020 ppm in strawberries); (7) monocrotophos average recovery ranged from 103.0% (0.5000 ppm in strawberries) to 110.2% (1.000 ppm in lettuce) and CVs ranged from 5.3% (1.000 ppm in lettuce) to 12.5% (0.3000 ppm in lettuce).

The data summarized above exclude 22 (3.5%) of the 630 reported results and were considered outliers. All of the α-BHC and acephate results (18 total) reported by Collaborator 4 were excluded. The HECD system used by this collaborator preferentially degraded the reference standard as evidenced by its ever-decreasing response with time. The degradation caused high results, which ranged from 100% recovery on the first quantitation to 250% recovery on the last quantitation. The acephate values reported by this collaborator were also excluded because they appeared consistently and comparatively low; the cause apparently was related to a standard preparation or degradation problem. Application of Dixon's test (11) for extremes indicated that 4 other values could be excluded with a P factor >95%.

Besides the α-BHC problem of Collaborator 4, relatively few major chromatographic problems were noted. Collaborator 6 reported that very poor resolution between acephate and omethoate was obtained on the DEGS column that was used for most of the study. Preparation of a second column, using the same required packing material as originally used, showed marked improvement in both the resolution and ability to quantitate the 2 chemicals. Collaborator 8 reported continually decreasing area responses for acephate and omethoate throughout the study and used injections of a cabbage extract, isolated per the method instructions, on the DEGS/FPD system to restore these responses to a higher sensitivity before they were quantitated. Several collaborators reported that p,p'-DDT (used in their performance check standard) degraded on their HECD system after 1 injection of the lettuce extract. Collaborators 3 and 4 did not approximate the recommended DEGS/FPD sensitivity of 50% full scale recorder deflection (FSD) for 6 ng monocrotophos and 1.5 ng chlorpyrifos. The monocrotophos response obtained by Collaborator 3 was half as sensitive (12 ng/50% FSD) and that obtained by Collaborator 4 was 7–10 times (40–60 ng/50% FSD) less sensitive when chlorpyrifos met the criterion of 1.5 ng for 50% FSD response.

The sensitivity of the method was determined by averaging randomly selected standard responses from those submitted by the collaborators (except monocrotophos from Collaborator 4) and calculating the average 50% FSD per amount

Table 8. Measures of precision for collaborative results of the Luke et al. method, using halogen-specific EC detection (Hall 700A) and methyl silicone column GC

Commodity	No.[a]	Pesticide	Added, ppm	Av. rec.,[b] ppm	$s_o{}^c$	$s_x{}^d$	$CV_o{}^e$	$CV_x{}^f$
Lettuce	1,2	α-BHC	1.044	1.063	0.0461	0.1115	4.3	10.5
	1,3	dieldrin	0.0844	0.0859	0.00867	0.0117	10.1	13.6
	1,3	chlorpyrifos	0.0636	0.0742	0.00925	0.0102	12.5	13.8
Tomatoes	2,3	dieldrin	0.1164	0.1142	0.0125	0.0172	11.0	15.1
	1,3	chlorpyrifos	0.6360	0.6488	0.0336	0.0398	5.2	6.1
Strawberries	2,3	α-BHC	1.044	1.026	0.0904	0.1198	8.8	11.7
	2,3	chlorpyrifos	0.3816	0.4350	0.0381	0.0552	8.8	12.7

[a] See Table 1.
[b] Outlying laboratories excluded from results.
[c] s_o is within-laboratory repeatability standard deviation.
[d] s_x is overall between-laboratory reproducibility.
[e] CV_o is coefficient of variation corresponding to s_o.
[f] CV_x is coefficient of variation corresponding to s_x.

Table 9. Measures of precision for collaborative results of the Luke et al. method, using phosphorus-selective FPD and DEGS column GC

Commodity	No.[a]	Pesticide	Added, ppm	Av. rec.,[b] ppm	$s_o{}^c$	$s_x{}^d$	$CV_o{}^e$	$CV_x{}^f$
Lettuce	1,3	chlorpyrifos	0.0636	0.0718	0.00586	0.00805	8.2	11.2
	1,2	acephate	10.0	9.153	0.622	0.679	6.8	7.4
	2,3	omethoate	2.008	2.130	0.132	0.210	6.2	9.9
	1,2	monocrotophos	1.000	1.090	0.0421	0.0764	3.9	7.0
Tomatoes	1,3	chlorpyrifos	0.636	0.6775	0.0431	0.0505	6.4	7.4
	1,2	acephate	4.00	3.516	0.249	0.272	7.1	7.7
	1,3	omethoate	1.004	1.101	0.0785	0.0949	7.1	8.6
	1,3	monocrotophos	0.500	0.534	0.0433	0.0473	8.1	8.9
Strawberries	2,3	chlorpyrifos	0.3816	0.4012	0.0285	0.0390	7.1	9.7
	1,3	acephate	0.500	0.4133	0.0294	0.0481	7.1	11.6
	1,3	omethoate	0.5020	0.4990	0.0339	0.0470	6.8	9.4
	1,2	monocrotophos	0.3000	0.3193	0.0235	0.0316	7.4	9.9

[a-f] See Table 8.

injected. For the DEGS/FPD systems, these values were 1.4 ng chlorpyrifos, 3.7 ng acephate, 6.8 ng omethoate, and 5.9 ng monocrotophos. For the methyl silicone/HECD systems, the 50% FSD responses were 0.5 ng α-BHC, 1.4 ng chlorpyrifos, and 1.1 ng dieldrin. Based on these average responses and assuming 10% FSD to be the lowest reliable detector signal for quantitation, the method, with 12 mg equivalent sample injections, gave limits of quantitation at 0.008 ppm α-BHC, 0.02 ppm chlorpyrifos and dieldrin, 0.06 ppm acephate, 0.10 ppm monocrotophos, and 0.12 ppm omethoate.

All collaborators reported that the unfortified samples of strawberries contained residues of endosulfans I, II, and SO_4 ranging from 0.1 to 0.3 ppm total, and that unfortified tomatoes contained methamidophos ranging from 0.08 to 0.34 ppm. No statistical treatment of these data was attempted because some collaborators reported only quantitative estimates from standards used for identification purposes. In addition to the endosulfans, the strawberries were reported to contain traces (<0.01 ppm) of α-BHC, dieldrin, and/or p,p'-DDE, as well as a significant halogen response that was not readily identifiable by the GC system specified for the study. This response was detected at an average retention time (relative to chlorpyrifos) of 3.3 on the methyl silicone column and was tentatively identified by Collaborators 5 and 9 as p,p'-dicofol. None of these reported residues or responses were considered to contribute to the recovery data reported. Contribution from the unfortified lettuce to the duplicate low level chlorpyrifos fortifications at 0.0636 ppm probably occurred. The lettuce contained a trace (<0.01 ppm) of chlorpyrifos, and no attempts were made to determine a quantitative value. At the fortification level, a 0.005 ppm blank would contribute approximately 8% to the total detected.

Measures of precision obtained for this study were based on the 19 sets of blind duplicate values which are summarized in Tables 8 and 9. The within-laboratory standard deviations and the corresponding CVs for each duplicate set were compared to overall between-laboratory standard deviations and their CVs. In all but 1 set of data, 1.044 ppm α-BHC in lettuce, the largest contribution of between-laboratory reproducibility of the method was from the variation in repeatability from within the laboratories.

Comments and Recommendations

Collaborator 5 used boiling chips as an aid in the solvent evaporation step and suggested that this should be a required part of the procedure. Boiling aids (chips) are not necessary if the solvent mixtures are slowly brought to boiling. However, the addition of boiling chips should not be discouraged from a safety standpoint, and they should not affect the performance of the method.

Several collaborators noted that the strawberries used in this study demonstrated atypical background residues and should not have been used. Preferably, these particular strawberries would not have been chosen, but availability at the time of preparation dictated their use. Pre-analysis and the results of the study itself showed that the background they provided did not affect the recoveries.

The recovery data, reproducibility, and repeatability demonstrated in this study compare favorably with past multiresidue method studies (12–16) that included similar (or identical) pesticides and commodities. Based on these satisfactory findings, it is recommended that this comprehensive procedure, featuring no cleanup of the acetone extract and the 2 separate GC systems, be adopted official first action for mul-

tiresidue determination of the 6 pesticides in the commodities studied. It is also recommended that this comprehensive procedure be investigated further in an attempt to include other chromatographic systems, commodities, and pesticides that may be adaptable to the no-cleanup approach.

Acknowledgments

References

(1) Luke, M. A., Froberg, J. E., & Masumoto, H. T. (1975) *J. Assoc. Off. Anal. Chem.* **58**, 1020–1026

(2) Mills, P.A., Onley, J. J., & Gaither, R. A. (1963) *J. Assoc. Off. Agric. Chem.* **46**, 186–191

(3) Storherr, R. W., Ott, P., & Watts, R. R. (1971) *J. Assoc. Off. Anal. Chem.* **54**, 513–516

(4) Holden, E. R. (1973) *J. Assoc. Off. Anal. Chem.* **56**, 713–717

(5) Luke, M. A., Froberg, J. E., Doose, G. M., & Masumoto, H. T. (1981) *J. Assoc. Off. Anal. Chem.* **64**, 1187–1195

(6) *Pesticide Analytical Manual* (1982) Vol. 1, Food and Drug Administration, Washington, DC

(7) *Official Methods of Analysis* (1984) 14th Ed., AOAC, Arlington, VA, Chapter 29, General Method for Organochlorine and Organophosphorus Pesticide Residues

(8) Condal-Bosch, L. (1964) *J. Chem. Educ.* **41**, A235, passim

(9) *Composition of Foods* (1963) Agriculture Handbook No. 8, U.S. Department of Agriculture, Washington, DC

(10) *Code of Federal Regulations* (1982) Title 40, Part 180

(11) Dixon, W. J., & Massey, F. J. (1969) *Introduction to Statistical Analysis*, 3rd Ed., McGraw-Hill, New York, NY, pp. 328–330

(12) Krause, R. T. (1966) *J. Assoc. Off. Anal. Chem.* **49**, 460–463

(13) Gaul, J. (1966) *J. Assoc. Off. Anal. Chem.* **49**, 463–467

(14) Davidson, A. W. (1966) *J. Assoc. Off. Anal. Chem.* **49**, 468–472

(15) Krause, R. T. (1973) *J. Assoc. Off. Anal. Chem.* **56**, 721–727

(16) Laski, R. R. (1974) *J. Assoc. Off. Anal. Chem.* **57**, 930–933

Residue Methodology for AMDRO Fire Ant Insecticide (AC 217,300) in Pasture Grass and Crops

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Residue methodology is described for the determination of AC 217,300 residues in pasture grass and crop samples. After extraction and subsequent cleanup on an XAD-2 column, residues of AC 217,300 are determined by liquid chromatography (LC), using a reverse phase paired-ion chromatographic system and detection at 300 nm. The method has a validated limit of sensitivity of 0.05 ppm with corresponding control values for the commodities analyzed of <0.01 ppm. Apparent residues over 0.05 ppm can be confirmed by either gas chromatography with an electron capture detector (GC-EC) or gas chromatography–negative ion chemical ionization mass spectrometry (GC-NICI). The direct GC-NICI method circumvents the need for sample cleanup on the XAD-2 column, and offers a greatly simplified procedure that is useful for screening samples. Recoveries of AC 217,300 from the commodities analyzed have been satisfactory with all methods of analysis.

AMDRO[1] fire ant insecticide has been developed to control imported fire ants. The active ingredient, AC 217,300 (Figure 1), is formulated as a 0.88% bait on pregel corn grits containing 30% w/w soybean oil as an attractant. In this bait medium, AC 217,300 is rapidly photodegraded ($T_{1/2}$ = 19 h of daylight) to many fragments, none of which is major. Stout et al. (1) have recently reported the mass spectrometric identification of the water photolysis products of AC 217,300, and Mallipudi et al. (2) have reported the rate of photodegradation and percent distribution of the photoproducts. Metabolism studies using carbon-14 benzylic or pyrimidine labeled AC 217,300 have shown that plants do not take up radioactivity from either the parent or its metabolites even at 10× rates. Hence, the only mechanism that allows occurrence of residues on plants or fruits is lodging of the bait on the commodity. Rate of disappearance studies show rapid elimination of AC 217,300 residues to below the 0.05 ppm tolerance level before the recommended 7-day withdrawal period.

This work was intended to develop appropriate residue methodology specific for AC 217,300 in pasture grass and a variety of crop samples.

METHOD

Special Notes

Rinse all clean glassware thoroughly with methanol and let dry before using. All solvents should be distilled in glass, suitable for pesticide analyses, as supplied by Burdick and Jackson Laboratories, Inc., or equivalent. AC 217,300 is light-sensitive, and in dilute solution it undergoes degradation. Therefore, all solutions containing the compound must be protected from light in all steps of the method. All glassware should be of low actinic variety (red or amber). If this is not available, other glassware may be covered with aluminum foil or flat black spray paint.

Reagents and Apparatus for LC

(a) *AC 217,300 standard solution.*—Prepare solutions containing 0.1, 0.2, 0.5, and 1.0 μg AC 217,300/mL LC mobile phase.

(b) *LC mobile phase.*—Dissolve 1.1 g heptanesulfonic acid sodium salt in 200 mL distilled water in 1 L volumetric flask. Add 10 mL glacial acetic acid and dilute to volume with
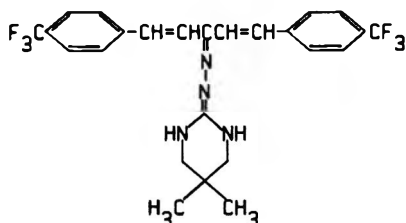
Figure 1.   Chemical structure of AC 217,300.

methanol. Degas solution by bubbling helium through it for about 30 s.

(c) *XAD-2 column.*—Place glass wool plug at bottom of a 19 × 300 mm Chromaflex chromatographic column equipped with 250 mL reservoir and Teflon stopcock (Kontes Glass Co., Vineland, NJ 08360; amber-coated columns are available on special order). Pour 50 mL hexane into tube. Prepare slurry of purified Amberlite XAD-2 resin (Applied Science Div., Milton Roy Co. Laboratory Group, PO Box 440, State College, PA 16801) in hexane. Add sufficient slurry to tube to give column height of 20 cm. Draw off solvent to within 1 cm of top of packing. If XAD-2 particles adhere to glass sides of column, wash down with a few mL methylene chloride. Place glass wool plug on top of packing.

(d) *Liquid chromatograph.*—Tracor Model 950 equipped with Tracor Model 970A variable wavelength detector, or equivalent system, suitable for use with stainless steel columns and capable of maintaining solvent flow of 2 mL/min and pressures up to 3000 psi. Instrument must be equipped with a sample injection valve capable of delivering 100 µL sample solution. Use an LC column (stainless steel, ¼ in. od, 4.6 mm id × 25 cm) packed with microparticulate silica (10 µm) to which is bonded an octadecyl moiety; Whatman Partisil PXS-1025-ODS-2, or equivalent, is suitable. LC operating conditions: column temperature ambient; mobile phase flow rate 1.5–2.0 mL/min; detector wavelength 300 nm; volume injected 100 µL; retention time for AC 217,300 ca 10 min.

(e) *Food chopper.*—Hobart.

(f) *Blender.*—Waring.

Reagents and Apparatus for GC-EC Confirmation

(a) *AC 217,300 standard solution.*—Prepare solutions containing 10, 20, and 100 ng AC 217,300/mL in mixture of hexane–2-propanol–methanol (85 + 5 + 10).

(b) *1% lard solution.*—Dissolve 1 g lard in 100 mL hexane–2-propanol–methanol (85 + 5 + 10). Use this solution to condition GC system.

(c) *Gas chromatograph.*—Hewlett-Packard Model 5730A, or equivalent with nickel-63 electron capture detector, suitable for on-column injections and fitted with 3 ft × 2 mm id glass column packed with 3% OV-17 on 60–80 mesh Gas Chrom Q (Applied Science Div., Milton Roy Co. Laboratory Group). Operating conditions: injector 300°C; column oven 235°C; detector 300°C; argon–methane (95 + 5) carrier gas at 25 mL/min; detector purge flow rate same as carrier flow; retention time for AC 217,300 ca 5 min.

Reagents and Apparatus for GC-NICI

(a) *AC 217,300 standard solution.*—Use standard solutions prepared in *Reagents and Apparatus for GC-EC Confirmation.*

(b) *1% lard solution.*—See *Reagents and Apparatus for GC-EC Confirmation.*

(c) *Methane.*—UHP, 99.97% minimum purity (Matheson Gas Products, East Rutherford, NJ 07073).

(d) *Gas chromatograph.*—Finnigan Model 9610, or equivalent, suitable for on-column injections and fitted with 4 ft × 2 mm id glass column packed with 3% OV-1 on 60–80 mesh Gas Chrom Q (Applied Science Div., Milton Roy Co. Laboratory Group). Operating conditions: injector 300°C; column oven 280°C; methane carrier gas at 15 mL/min; retention time for AC 217,300 ca 2 min.

(e) *Mass spectrometer.*—Finnigan Model 4000 equipped with Pulsed Positive Ion Negative Ion Chemical Ionization (PPINICI) accessory, or equivalent system. Operating conditions: GC-MS interface temperature 300°C; ion source temperature 350°C; methane source pressure 0.4 torr; conversion dynodes ± 3000 V; electron multiplier 1000 V; preamplifier range 10^{-8} amp/V.

(f) *Data system.*—INCOS Model 2300, or equivalent. Ions at m/z 492⁻ and 494⁻ were monitored with a dwell time of 400 ms/ion.

Procedure for LC

Pulverize dry ice in bowl of Hobart food chopper to chill bowl and grater thoroughly. Attach 9 in. vegetable slicer attachment with ³⁄₃₂ in. shredder plate and chop tissue to fine particle size. Continue adding sample and chopping until complete sample is chopped. If necessary, add more dry ice to keep sample frozen during chopping procedure. Store sample in freezer (−20°F) until dry ice has dissipated.

Transfer representative 20 g sample of commodity to Waring blender jar and blend 5 min with 200 mL methylene chloride. Filter by vacuum into Buchner funnel through ¼ in. thick precoat of Celite 545 AW placed on glass-fiber paper. Dry filtrate by swirling with ca 20 mL (measured in beaker) anhydrous sodium sulfate.

Decant 50 mL clear filtrate and transfer to 125 mL Erlenmeyer flask. Add 50 mL hexane to flask and swirl contents to mix. Pass solution through XAD-2 column, setting stopcock to give flow of ca 6–8 drops/s. Do not let the column run dry! Wash column with 150 mL methylene chloride. Place clean 500 mL round-bottom flask under column, and elute column with 100 mL acetone followed by 100 mL methanol. (Column may be regenerated by washing with 50 mL acetone followed by 150 mL hexane). Place flask containing eluate on rotary evaporator and, using 25–35°C water bath, evaporate solution to ca 20 mL. Quantitatively transfer solution to 100 mL pear-shape flask by using few mL acetone. Place flask on rotary evaporator and evaporate contents to dryness. Dissolve residue in exactly 2 mL LC mobile phase, giving equivalent of 2.5 g commodity/mL ($Conc_{comm}$). Inject 100 µL aliquots onto chromatographic column and measure response (peak height or area) of AC 217,300 in sample (R_{samp}) against response of external AC 217,300 standard (R_{std}). Concentration of AC 217,300 in commodity equals (R_{samp}/R_{std}) × ($Conc_{std}/Conc_{comm}$).

Procedure for GC-EC Confirmation

If LC of field-treated sample indicates apparent AC 217,300 residue of >0.05 ppm, identity of LC peak should be confirmed by GC. Re-inject 100 µL aliquot of sample and collect, in 125 mL separatory funnel, solvent eluting at retention volume of apparent AC 217,300 peak. Add 10 mL 10% aqueous sodium carbonate solution to funnel, mix well, and add 25 mL methylene chloride. Shake funnel vigorously for 20 s and let phases separate. Draw methylene chloride layer into 100 mL pear-shape flask, and repeat partitioning step with another 25 mL methylene chloride. Evaporate combined methylene chloride layers to dryness on rotary evaporator using 25–35°C water bath. Dissolve residue in exactly 1 mL hexane–

methanol–isopropanol (85 + 10 + 5 by volume) to give equivalent of 0.25 g of commodity per mL (Conc_comm).

Before starting GC-EC confirmatory analyses, be sure GC column is properly conditioned. Inject 5 μL lard solution, wait until baseline stabilizes, and then inject 5 μL standard AC 217,300 (20 ng/mL). Repeat alternate injections of lard and standard solutions until good peak shape is obtained. Continue injection of standards until constant response is obtained. When GC system is properly conditioned, inject 5 μL aliquots onto chromatographic column and measure response of AC 217,300 in sample (R_{samp}) against response of external AC 217,300 standard (R_{std}). Calculate concentration of AC 217,300 in commodity by equation given in *Procedure for LC*.

Procedure for GC-NICI

Chop and extract commodity as described in *Procedure for LC*. Dry extract with sodium sulfate, then decant 100 mL clear filtrate and transfer to a 250 mL round-bottom flask. Connect flask to rotary evaporator. Evaporate solvent in 25–35°C water bath until volume of extract is somewhat less than 25 mL. Do not evaporate extract to dryness! Transfer extract to 25 mL low-actinic glass volumetric flask. Rinse round-bottom flask with small amount of methylene chloride and combine rinse with extract in volumetric flask. Add methylene chloride to volumetric flask to bring up to volume, yielding equivalent of 0.40 g commodity/mL (Conc_comm).

Before starting GC-NICI analyses, be sure GC injection port is properly conditioned by checking ratio of m/z 492⁻ ion to m/z 494⁻ ion on 5 μL injection of GC working standard (20 ng/mL). If injection port is not properly conditioned, most AC 217,300 injected will be transformed into another compound that elutes 10–15 s sooner and generates ion at m/z 492⁻. Inject 5 μL aliquots of 1% lard solution with GC effluent diverted from ion source until peak height of m/z 492⁻ ion is <20% of peak height of AC 217,300 measured at m/z 494⁻. When injection port is properly conditioned, inject 5 μL aliquots of sample extract and measure AC 217,300 content against external AC 217,300 GC working standard in same manner as described in *Procedure for GC-EC Confirmation*.

Results and Discussion

Development of a satisfactory residue method specific for AC 217,300 was an extremely difficult project. The main reasons were the inapplicability of conventional cleanup procedures, the binding of AC 217,300 to the dried crude residue if initial extracts were evaporated to dryness, and the photoinstability of AC 217,300. The latter 2 problems were overcome by avoiding the initial stripping step and storing all extracts in glassware suitable for avoiding light penetration.

The complete failure of conventionally used cleanup procedures for isolation of the analyte from chlorophylls, carotenes, etc., in green foliage presented the most difficult problem. The typically used Florisil or silica gel column chromatographic procedures as well as slurrying with Darco were of essentially no use because AC 217,300 was bound tightly to all these adsorbents. Even the classically used deactivation procedures failed because at lower residue levels (<0.1 ppm) 50–60% of the AC 217,300 in the plant extracts was bound to the adsorbent. Work with carbon-14 labeled AC 217,300 showed that the compound was bound vs a decomposition or ultimate determination problem.

In the early stages of method development, an instrumental method for reliably measuring residue levels of AC 217,300 also presented problems. Satisfactory electron capture response could be attained from a gas chromatograph equipped

with a nickel-63 EC detector and a 3% OV-1 column. In the early stages of method development on pasture grasses, however, conditioning the GC column to achieve reproducible and satisfactory response in addition to one reasonably shaped peak was elusive. Later, during simultaneous efforts to develop a residue method for the assay of cattle tissues, we observed that several injections of cattle muscle or fat extracts conditioned the GC system, resulting in improved peak shape and more reproducible response. It was quickly demonstrated with freshly packed columns that an injection of 1% lard solution served the same purpose. Consequently, this solution was used for later GC investigations and was required for this methodology, as noted in the direct GC-NICI method.

Hence, the first residue methodology for the analysis of pasture grasses consisted of partial cleanup of the methylene chloride extract (diluted 1:1 with *n*-hexane) on an XAD-2 column, final cleanup by using LC on a Whatman Partisil PXS-1025-PAC column with a hexane–2-propanol–methanol (85 + 5 + 10) solvent system, collection of the fraction containing AC 217,300, and final determination of the AC 217,300 by GC-EC. However, because this methodology was exceedingly long and tedious, alternative approaches were explored.

Attempts at performing a direct determination of AC 217,300 by LC were stymied by inadequate response and interfering peaks, using the aforementioned mobile phase and fixed wavelength UV detection at either 254 or 280 nm. With the subsequent availability and acquisition of a variable wavelength UV detector operated at the 300 nm absorbance maximum of AC 217,300, the response of AC 217,300 for direct LC determination was greatly enhanced. The development of a reverse phase paired ion chromatographic (PIC) system using heptanesulfonic acid satisfactorily removed naturally occurring interferences from all of the plant extracts examined. Many other pesticides were shown not to elute at the same retention time as AC 217,300. These included methoxychlor, aldrin, dieldrin, BHC, lindane, azinphos methyl, carbaryl, endrin, and parathion. With a 100 μL injection aliquot equivalent to 250 mg of the commodity, the linear response range of 10–100 ng was equivalent to 0.04–0.40 ppm AC 217,300 in the commodity. Table 1 reports recovery values of AC 217,300 from various commodities, which were obtained from laboratory fortification studies. Figure 2 shows typical LC chromatograms for leaf lettuce fortified at 0.05 ppm and control leaf lettuce processed through the procedure.

Table 2 summarizes the confirmatory data obtained by GC-EC, and Figure 3 shows typical chromatograms of control and fortified leaf lettuce. The GC-EC method was linear from 50 to 500 pg of injected standard corresponding to the same concentration range as the LC method.

A second alternative approach to the determination of AC 217,300 residues was a direct GC-NICI method. Exceptional behavior of AC 217,300 by GC-NICI was anticipated on the basis of reports by Hunt et al. (3), Hunt and Crow (4), and Stout and Steller (5), using GC-NICI for strongly electronegative compounds. By taking advantage of the tremendous EC response of AC 217,300, the high molecular weight of AC 217,300, the generation of almost solely the M⁻ of AC 217,300 in NICI(CH₄), and the mass specificity of a mass spectrometric detector, a greatly simplified sample preparation procedure was feasible. Sample workup before GC-NICI analysis required only extracting the commodity with methylene chloride, drying the extract with sodium sulfate, and concentrating the extract. By selectively monitoring the m/z 494⁻ ion, the M⁻ of AC 217,300, GC-NICI demonstrated a linear

Table 1.   Recovery of AC 217,300 from commodities determined by LC procedure

Commodity	Control, ppm	Fort. level, added,ppm	Av. % rec. (No. of replicates)	Range, %
Alfalfa	<0.009	0.05	84.5 (3)	80.0–88.9
		0.10	74.9 (3)	66.7–80.0
		0.20	80.2 (2)	77.5–82.9
		0.50	65.0 (1)	
		1.00	80.5 (1)	
Overall av. (std dev.)			78.4 (7.4)	
Leaf lettuce	<0.008	0.05	90.0 (4)	88.0–96.0
		0.10	80.2 (4)	68.6–88.4
		0.20	84.9 (2)	77.9–92.0
		0.50	76.8 (1)	
		1.00	75.5 (1)	
Overall av. (std dev.)			83.6 (7.9)	
Broccoli	<0.010	0.05	85.7 (4)	80.0–102.9
		0.10	95.1 (3)	90.0–100.0
		0.20	77.4 (3)	65.9–91.4
		0.50	75.5 (2)	75.0–76.1
		1.00	89.7 (1)	
Overall av. (std dev.)			84.7 (11.0)	
Soybean foliage	<0.004	0.05	90.6 (3)	77.8–106.5
		0.10	80.5 (3)	70.5–91.5
		0.50	88.0 (1)	
		1.00	80.2 (2)	75.3–80.2
Overall av. (std dev.)			84.5 (10.6)	
Bahia grass	<0.010	0.05	83.3 (4)	75.3–97.8
		0.10	73.7 (5)	62.2–89.4
		0.20	69.0 (2)	68.0–70.0
		0.50	76.6 (4)	69.6–87.8
		1.00	67.8 (5)	62.8–73.3
Overall av. (std dev.)			74.2 (9.5)	
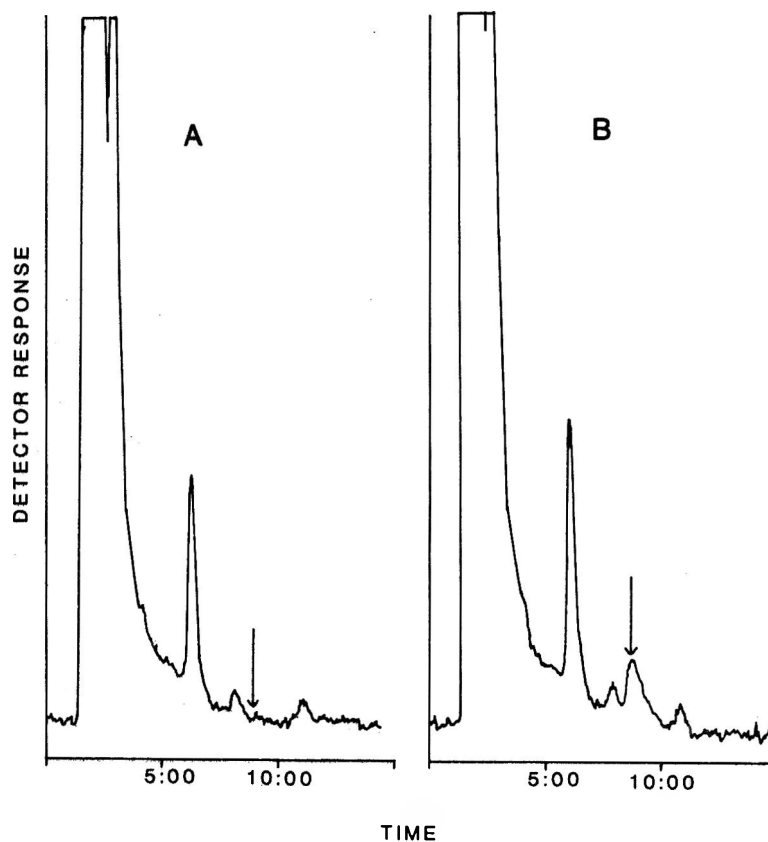
Figure 2.   LC chromatograms of (A) control leaf lettuce and (B) leaf lettuce fortified at 0.05 ppm. Arrow shows AC 217,300 peak.

Table 2. Recovery (%) of AC 217,300 from commodities determined by GC-EC confirmatory procedure

Commodity	Control, ppm	Fort. level, added, ppm			
		0.05	0.10	0.20	0.50
Alfalfa	<0.015	101.8	94.5	81.8	—[a]
Leaf lettuce	<0.012	86.2	80.0	76.9	—
Broccoli	<0.012	98.5	98.5	89.2	—
Soybean foliage	<0.001	92.1	88.6	—	90.8

[a]Recovery experiment not performed at this fortification level.

Table 3. Recovery (av. %) of AC 217,300 from commodities determined by GC-NICI procedure

Commodity	Control, ppm	Fort. level, added, ppm	
		0.05	0.1
Bahia grass	0.001	80.9	79.6
Alfalfa	<0.001	60.4	50.1
Soybean foliage	<0.001	70.9	71.7
Broccoli	<0.002	80.3	80.5
Corn silage	0.001	90.6	85.9
Leaf lettuce	0.001	119.6	105.4
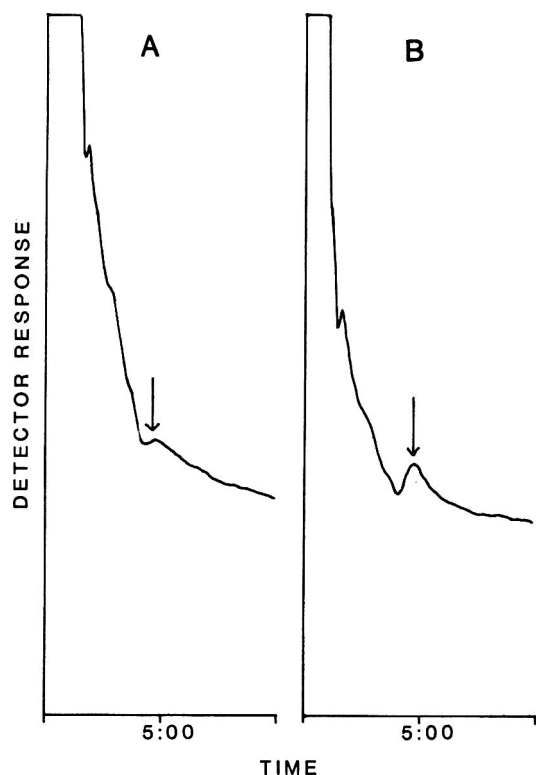
Figure 3. GC-EC chromatograms of (A) control leaf lettuce and (B) leaf lettuce fortified at 0.05 ppm. Arrow shows AC 217,300 peak.

Figure 4. Ion maps from GC-NICI analyses of (A) 100 pg AC 217,300 standard, (B) control leaf lettuce, and (C) leaf lettuce fortified at 0.05 ppm AC 217,300.

response range of 40–400 pg. For an injection aliquot corresponding to 2 mg of the commodity, this response range was equivalent to 0.02–0.20 ppm AC 217,300 in the commodity.

Figure 4 compares the analyses of a 100 pg standard (corresponding to 0.05 ppm AC 217,300 in the commodity), an extract of control leaf lettuce, and an extract of leaf lettuce fortified at 0.05 ppm. Table 3 shows that control extracts of the commodities analyzed had apparent AC 217,300 residues of <0.002 ppm. Table 3 also shows that recoveries were acceptable with most commodities. Instances of low recoveries could be overcome by use of a carbon-13 labeled internal standard.

In conclusion, the final residue method for AC 217,300 in pasture grass and crop samples uses LC with a reverse phase PIC system and detection at 300 nm. Residues over the tolerance of 0.05 ppm are not expected as discussed in the introduction, so apparent residues over this level in field-treated crops should be confirmed by either GC-EC or the direct GC-NICI method. Although it requires more sophisticated instrumentation, the direct GC-NICI method offers a

greatly simplified cleanup procedure and a rapid sample turnaround that is useful for screening purposes.

REFERENCES

(1) Stout, S. J., Mallipudi, N. M., Lee, A., & Orloski, E. J. (1984) *Biomed. Mass Spectrom.*, submitted for publication
(2) Mallipudi, N. M., Lee, A., Stout, S. J., & Orloski, E. J. (1984) *J. Agric. Food Chem.*, manuscript in preparation
(3) Hunt, D. F., Stafford, G. C., Crow, F. W., & Russell, J. W. (1976) *Anal. Chem.* 48, 2098–2105
(4) Hunt, D. F., & Crow, F. W. (1978) *Anal. Chem.* 50, 1781–1784
(5) Stout, S. J., & Steller, W. A. (1984) *Biomed. Mass Spectrom.* 11, 207–210

Improved Polarographic Method for Determination of Glyphosate Herbicide in Crops, Soil, and Water

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A prior method for determination of glyphosate in water samples has been modified to accommodate samples of crops and soils. Differential pulse polarography as the determinative step enables analysis in an aqueous medium, which is important during extraction of this compound. Residues are cleaned up and concentrated by ion exchange on a strong basic resin in OH⁻ form. The method is rapid, is applicable to a relatively broad selection of sample types, and gives recoveries consistently better than 60% with good precision. The main shortcoming of the method is that the limit of detection of 0.5–1.0 ppm may sometimes be inadequate. The metabolite, aminomethyl phosphonic acid, is not detected.

Analysis of the important herbicide glyphosate has been reviewed recently by Guinivan et al. (1). They presented a modification of the gas chromatographic method (2) originally developed by the Monsanto Co. and appearing in the FDA *Pesticides Analytical Manual* (PAM). More recently, Moye et al. (3) published a simplified liquid chromatographic method. However, a faster, reasonably sensitive, and generally more applicable procedure is still needed. We decided to improve our prior polarographic method (4).

The improved method has several steps in common with the PAM procedure (2) but is more precise and considerably faster, because time-consuming evaporations as well as the use of organic solvents have been eliminated. The determinative step is differential pulse polarography of the reducible derivative, nitroso-glyphosate, which is prepared directly in the electrolyte solution for later transfer to the polarographic cell.

METHOD

Apparatus

(a) *Polarograph.*—Princeton Applied Research, Model 174A, with M174/70 drop timer assembly. The instrument was operated in 3-electrode mode, using dropping mercury working electrode, silver wire (0.7 mm) as auxiliary electrode, and AgCl-coated silver wire as quasi-reference electrode (e.g., see ref. 5). A 30 mL Teflon screw-cap vial ("Tuf-Tainer," Pierce Chemical Co., Rockford, IL) was used as polarographic cell (see Figure 1). The screw cap was clamped to a rack and served as holder for the cell and, consequently, needs a center hole for the DME-assembly wide enough to permit free drop release motion (important!) and a smaller hole for the purge gas tubing.

(b) *Chromatographic columns.*—200 mm × 25 mm id with Teflon stopcock, sintered-glass (G1) disk, and 250 mm × 70 mm id reservoir on top.

(c) *Glass fiber filter.*—Whatman GF/F, 4.7 cm, or equivalent.

Reagents

(a) *Anion-exchange resin, Dowex 1-X8.*—100–200 mesh, chloride form (Sigma Chemical Co., St. Louis, MO). Convert to hydroxide form by adding 1N KOH to cover resin in original plastic bottle overnight or longer. Transfer slurry to

chromatographic column and wash with water to neutral pH. Store as water slurry.

(b) *Cation-exchange resin, Dowex 50-X8.*—100–200 mesh, hydrogen form. Use as received.

(c) *Sodium nitrite solution.*—1% aqueous. Prepare fresh daily.

(d) *Ammonium sulfamate solution.*—10% aqueous. Stable for months at room temperature.

(e) *Glyphosate standard solution.*—Dissolve by heating 100 mg glyphosate analytical standard (Monsanto Co., St. Louis, MO) in 100 mL water. Stock solution is stable for years at room temperature.

Procedure

A schematic of procedure is shown in Figure 2.

Extraction

(a) *Soil.*—To 50 g homogenized soil in 500 mL centrifuge vessel, add 100 mL 0.5N KOH. Close vessel with rubber stopper and shake on mechanical shaker 15 min. Remove stopper and, depending on centrifuge available, spin 10–30 min (10 min if centrifuge can exceed 4000 rpm) and decant as much of clear, dark brown liquid as possible into 400 mL bottle. Add another 100 mL 0.5N KOH to centrifuge vessel. Break up and stir sediment with glass rod, and shake again 15 min. Centrifuge and add supernate to first extract portion. Fill bottle to 400 mL mark with water and proceed to anion-exchange step.

(b) *Crops.*—To 25 g homogenized crop sample in Waring blender, add 250 mL water and 20 mL CH₂Cl₂ and mix 3 min. Transfer total contents to 500 mL centrifuge vessel and centrifuge 30 min at ≥ 3000 rpm. Using 100 mL syringe or by decantation, carefully separate clear supernate from CH₂Cl₂-crop residue and transfer supernate to 500 mL beaker containing 30 g cation-exchange resin. Sonicate beaker 10 min in ultrasonic bath.

Filter by suction through glass fiber filter, and wash resin that is present on filter with 250 mL water. Adjust combined filtrates and washings to pH 10–11 with KOH solution.

Anion exchange

Prepare column by loading with 20 mL settled resin. Wash resin down from inner wall with water to 4–5 cm height above filter disk. To prevent whirling of resin, press wad of glass wool on the top.

Near end of working day, add water sample or soil or crop extract to prepared column. With stopcock, adjust flow to maximum of 2 drops/s. If flow decreases, open stopcock completely. Let column run overnight or weekend. At this and later steps, column may run empty. Next day, wash empty column with 50 mL 0.25N KOH and, when solution has drained, wash with 50 mL water. Discard percolate and washings, and place 100 mL bottle containing 0.5 g activated charcoal and 10 mL 5N HCl under column outlet. At flow ≤ 0.5 drop/s, elute column with 40 mL 1N HCl. If flow ceases (due to development of CO₂ pockets), push down on glass wool with glass rod, possibly combined with regulation of stopcock. Tumble or shake eluate in bottle 15 min, and remove charcoal by means of glass fiber filter. Transfer 25 mL filtrate

Figure 1. Polarographic cell.

GLYPHOSATE DETERMINATION   SCHEMATIC

Figure 2. Schematic illustration of procedure.

to 50 mL glass bottle with screw cap. Save rest of filtrate to serve as a control for polarographic background.

Nitrosation

From stock solution of glyphosate, prepare 2 duplicate reference standards containing 25 μg and 100 μg (or more if samples are expected to contain excessive residue levels) in 25 mL 1N HCl for nitrosation, along with each series of samples. To 25 mL portions of samples and standards, add 1 mL sodium nitrite solution, put on screw caps, shake 1 s by hand, and leave bottles 15 min. Then destroy excess nitrite by adding 1 mL ammonium sulfamate solution.

The nitrosated derivative is stable for several days at room temperature.

Polarography

Pour ca 10–15 mL unnitrosated portion of sample (for standards, use 1N HCl) into polarographic cell. Displace oxygen from system by bubbling high-purity nitrogen through solution in cell 4 min. Remove nitrogen inlet tubing, and start polarographic measurement when current at starting voltage has stabilized (ca 1 min), using the following conditions: scan start, -0.5 V; rate, 2 mV/s in negative direction; pulse amplitude, 100 mV; drop time, 2 s. Repeat above procedure, using corresponding nitrosated sample, and start scan at same current level by adjusting offset control.

If this step of the procedure has been postponed to accumulate a reasonable number of samples before start of the polarography, base final calculation on standards that have paralleled the samples. To save time during a run of a series of samples, work with 2 Teflon vials and deaerate next sample during polarographic measurement. Purge time after change of container can then be reduced to 2 min.

Results and Discussion

We demonstrated application of the method for 2 different sample types. Figure 3 shows polarograms of nitrosated portions of extracts of blueberries, a type of crop which Guinivan et al. (1) stated to be difficult to analyze by the gas chromatographic method (2). By the present method, this matrix does not present problems, and glyphosate concentrations of 0.5 ppm or higher can easily be distinguished from untreated crop samples. It should be noted that in this case the sample weight was 50 g.
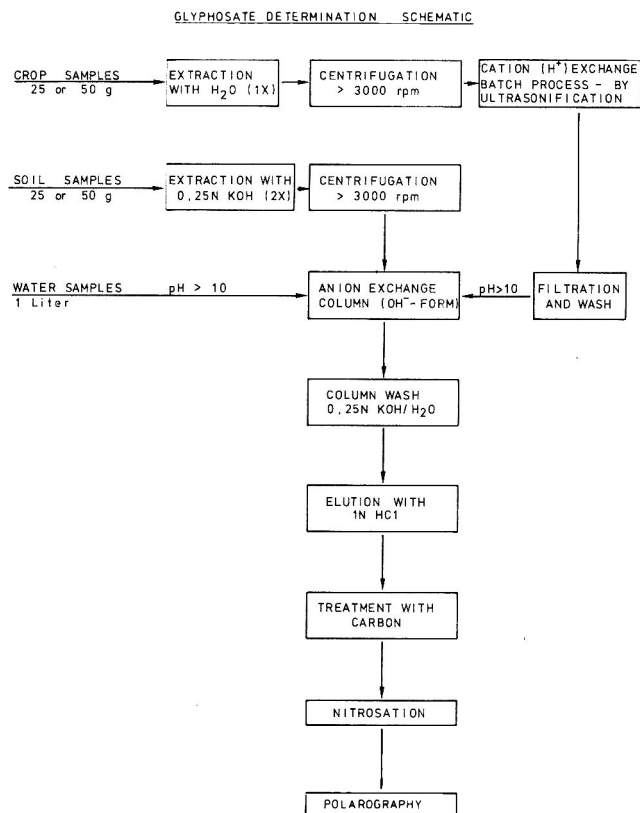
The series of polarograms shown includes 2 parallel reference samples to demonstrate the good reproducibility of the polarographic step (including nitrosation). The response curve for standards had been shown to be linear (4). For the present modifications the response is also linear over the range 25–2500 μg.

We experienced little instrumental trouble with the polarographic technique, especially after we changed to a quasi-reference electrode and omitted the salt bridge. The platinum counter electrode originally used had to be replaced with a silver electrode to avoid interference from an extra peak appearing shortly before the "hydrogen wall." This extra peak increased with increasing age of the platinum electrode, but has not been observed after the change to a silver electrode.

Using a small Teflon vial as polarographic cell for relatively small volumes of cleaned extracts is advantageous, because it drips dry when emptied and normally does not need washing between runs. In contrast to other plastic materials tested, Teflon does not retain nitrogen bubbles that may loosen during the polarographic process and disturb the mercury drop.

Figure 4 shows polarograms of a natural water sample containing glyphosate at 2 orders of magnitude less than the samples of Figure 3. It shows the recommended way of performing the polarographic step by preceding each polarogram of nitrosated extract with a corresponding run of the unnitrosated portion. Also indicated in this figure are the measurements used for quantitation of results, on the basis of the formula:

Glyphosate concentration (μg/L) =

$$(h_1 - h_0) \times 2 \times (A/H)$$

where the factor 2 indicates that only half the amount of total extract is used in the nitrosation step; and A and H represent the amount (μg) of glyphosate standard used as reference and
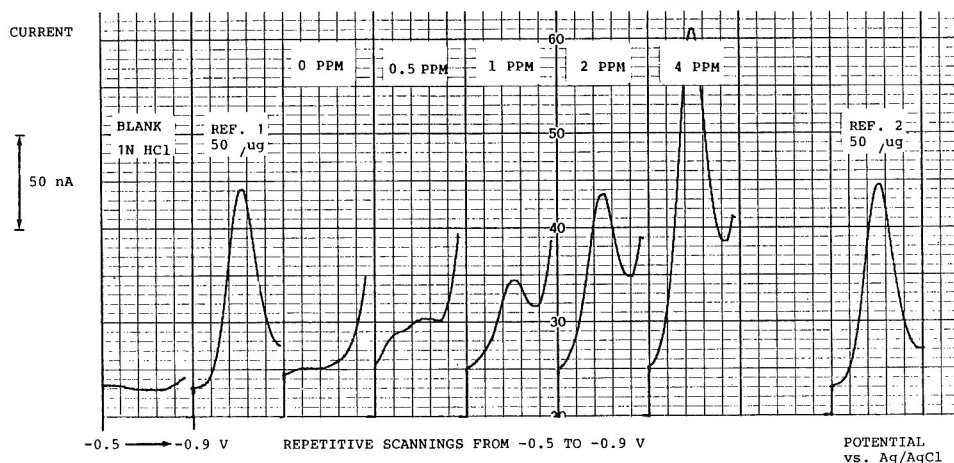
Figure 3.   Polarograms of glyphosate standard and extracts of 50 g samples of blueberries fortified with glyphosate.
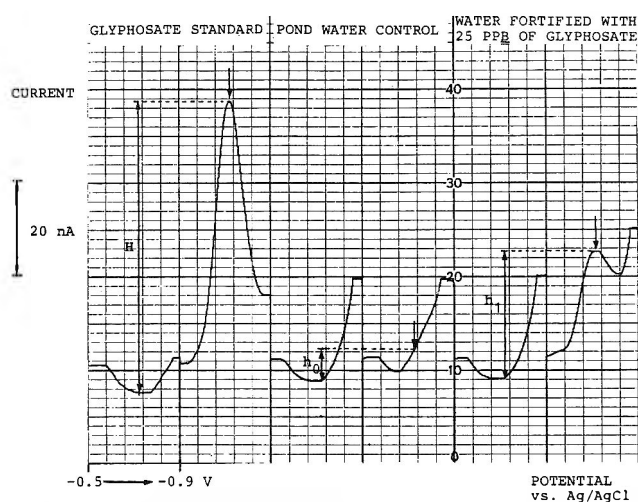
Figure 4.   Determination of low levels of glyphosate in water. Arrows point to peak potential of nitrosated glyphosate derivative. Every second scan area shows polarograms of nitrosated solutions. Preceding scan areas show polarograms of unnitrosated solutions.

Table 1.   Analysis of fortified materials for glyphosate

Sample	Fort., mg/kg	Rec., % Mean	Rec., % Range	n	CV, %
Berries:					
Blueberries	2.0	86	79–94	7	6
Cowberries	2.0	77	73–80	4	3
Raspberries	2.0	72	63–84	9	7
Cereal Grains:					
Barley	2.0	68	62–75	14	4
Oats	2.0	64	62–70	6	3
Wheat	2.0	66	63–68	4	3
Rice	2.0	63	62–64	2	—
Other Crops:					
Grass	2.0	70	65–73	3	5
Potato	2.0	80	77–83	2	—
Soils:					
Loam	1.0	70	63–75	4	5
Loam	2.0	86	83–89	4	3
Silt	4.0	73	67–78	5	6
Water:					
Tap	0.050	76	67–81	8	5
Pond	0.025	72	68–75	2	—

its corresponding peak height at peak-potential, respectively. The meaning of the other 2 symbols is indicated in Figure 4.

Because of the good reproducibility of the polarographic step, it usually is not necessary to run more than 2 references for each set of samples. If all samples for one day were nitrosated simultaneously, one reference at the beginning and one at the end would probably be adequate. In any case, the response factor should be calculated on the basis of the average height of the 2 most pertinent reference peaks.

If the detection limit is considered to be the concentration that leads to a plateau or shoulder at the peak potential of the nitrosated compound (see 4th scanning of Figure 3), 0.5 ppm would be the detection limit for blueberries, cowberries, and soils. For other substrates mentioned in Table 1, the cleanup procedure cannot handle sample weights > 25 g, and thus, 1 ppm is the limit of detection.

In the fortification studies, presented in Table 1, crop samples have been fortified before extraction with standard solutions of 2 ppm glyphosate; this is likely to become the maximum residue limit for the compound in this country. The average recovery at this level is 65% for cereal grains and 70% or better for other crops studied; no individual value was lower than 62%. Because the coefficient of variation within each matrix group is relatively low, acceptable results are obtainable by correcting for the procedural loss. The

loss probably occurs during the extraction, as a result of adsorption of glyphosate to the matrix, and/or during the anion cleanup step, due to interfering anions. The nitrosation process apparently is not influenced by foreign components remaining after cleanup. When cleaned extracts of barley and raspberries were fortified with glyphosate immediately before nitrosation, average recoveries of 102% and 93%, respectively, were obtained.

The technique of preceding each polarogram of nitrosoglyphosate with a recording of an unnitrosated part of the solution adds increased selectivity to the method, because this precaution makes it possible to detect any directly polarographic active compound, either naturally present in the sample or introduced along with the reagents, that may survive the cleanup step.

So far, no collaborative testing of the method has been initiated. In a few instances, however, results obtained have been compared with those derived from identical samples analyzed at Monsanto's Technical Center in Belgium, using their established chromatographic procedures. The samples were wild raspberries, which had been subjected to regular spraying in a forest area, and raw barley sampled from fields that had been sprayed shortly before harvest. Table 2 presents the results. All figures represent the average of duplicate determinations corrected for procedural losses. For the great

Table 2. Comparison of results obtained by 2 different methods[a]

Sample		Present method, ppm	Found by Monsanto, ppm
Raspberries	1	13.1	9.9
	2	13.7	12.9
Barley	1	4.8	5.1
	2	6.6	8.0
	3	13.8	13.7
	4	6.7	7.6
	5	9.7	10.0
	6	26.6	25.9
	7	13.4	14.0
	8	21.6	23.4
	9	15.5	16.1

[a]Average of duplicate determinations, corrected for procedural losses.

procedural difference between the 2 methods, the agreement of results is very acceptable.

According to the chromatographic determinations, these samples also contained residues of the metabolite aminomethyl phosphonic acid (up to a maximum of 0.16 ppm). The metabolite will not be detected by the present method; however, in most cases this probably will not be a severe drawback.

An important new feature of this improved method is that the anion-exchange cleanup/concentration is performed on a resin in OH^- form, which retains glyphosate better than a resin in the bicarbonate (2) or chloride (4) form, and which allows some interfering background compounds to be washed from it by 0.25N KOH.

The preliminary ion exchange on a strong cationic resin, used for crop extracts, decreases interferences from basic components amenable to nitrosation. This step could more conveniently be done as a batch process, speeded up by use of ultrasonification. In this way the total analysis time can be reduced to 5 h, assuming that percolation of the sample through the anion-exchange column takes place overnight.

Glyphosate has to be converted to a nitroso-derivative before polarography. There is some general toxicological concern about nitrosation reactions; such concern has been expressed, e.g., by Smyth et al. (6) and Young et al. (7) for glyphosate determination. The nitroso derivative of glyphosate has been subjected to an Ames test for mutagenicity and found to give no response (E. Dybing, National Institute of Public Health, Norway, 1979). In addition, there is practically no manipulation of the nitroso derivative because it is produced in the last step of the procedure. Also, at this stage, other nitrosable compounds have been removed because they interfere during polarography.

Acknowledgments

REFERENCES

(1) Guinivan, R. A., Thompson, N. P., & Wheeler, W. B. (1982) *J. Assoc. Off. Anal. Chem.* **65**, 35–39
(2) Food and Drug Administration (1977) *Pesticide Analytical Manual,* Vol. II, FDA, Washington, DC, section 180–364
(3) Moye, H. A., Miles, C. J., & Scherer, S. J. (1983) *J. Agric. Food Chem.* **31**, 69–72
(4) Brønstad, J. O., & Friestad, H. O. (1976) *Analyst* **101**, 820–824
(5) Sawyer, D. T., & Roberts, J. L., Jr. (1974) *Experimental Electrochemistry for Chemists,* John Wiley & Sons, New York, NY, p. 50
(6) Smyth, W. F. & Smyth, M. R. (1979) in *Polarography of Molecules of Biological Significance,* W. F. Smyth (Ed.), Academic Press, New York, NY, p. 31
(7) Young, J. C., Khan, S. U., & Marriage, P. B. (1977) *J. Agric. Food Chem.* **25**, 918–922

PRESERVATIVES AND ARTIFICIAL SWEETENERS

Extraction of Organic Acids by Ion-Pair Formation with Tri-n-Octylamine. Part 6. Determination of Sorbic Acid, Benzoic Acid, and Saccharin in Yogurt

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The sorbate content of commercial yogurt samples is determined by reverse phase liquid chromatography following ion-pair extraction with tri-n-octylamine. Mean recoveries (70–88%), precision (1.1–3.3% RSD), and detection limit of the method are presented for sorbic acid, benzoic acid, and saccharin.

The Belgian food legislation permits the addition of sorbic acid and its sodium, potassium, and calcium salts to yogurt with fruit. In August 1983, the maximum allowed concentration was decreased from 300 to 150 mg/kg (1). Other preservatives such as benzoic acid, however, are prohibited.

The analysis scheme we chose, consisting of an ion-pair extraction with tri-n-octylamine (TnOA) followed by liquid chromatographic (LC) determination, was already reported for the determination of synthetic dyes in several foods (2–4) and of dyes, preservatives, and saccharin in soft drinks (5). Because yogurt has a more complex composition than soft drinks, the original method had to be modified to liberate the additives from the food matrix. The applicability of the method is shown through recovery and precision studies and by the analysis of real samples. The presence of benzoic acid and saccharin, both prohibited in yogurt, was also investigated.

Experimental

Apparatus and Reagents

(a) *Apparatus.*—Varian LC 5060 equipped with manual Valco loop injector (loop size 100 μL), Varian UV 100 detector, and Varian Vista 401 integrator. pH values were controlled with an Orion Ionalyser Model 601 and a combined glass and calomel electrode.

(b) *Column.*—RP-18 (Merck, Darmstadt, GFR), 250 mm × 4 mm id, particle size 10 μm.

(c) *Mobile phase.*—Methanol–phosphate buffer (pH = 4.5, ionic strength = 0.1) (40 + 60). Mobile phase was filtered through a 0.45 μm filter (Millipore).

(d) *Reagents.*—Tri-n-octylamine. (Aldrich, Beerse, Belgium). Methanol, LC grade (Merck). Sodium phosphate, sodium benzoate, and potassium sorbate, analytical grade (Merck). Sodium saccharin (BDH, Poole, UK). Chloroform, analytical grade (Merck), saturated with water. Water was double-distilled.

(e) *Buffers.*—For 2 L and ionic strength 0.1. pH = 4.5: 0.9 mL 1M H_3PO_4 and 27.598 g $NaH_2PO_4.H_2O$. pH = 5.5: 24.650 g $NaH_2PO_4.H_2O$ and 1.260 g $Na_2HPO_4.2H_2O$.

(f) *Stock solutions.*—Potassium sorbate: 0.1, 0.5, 1, 2, and 3 mg/mL; sodium benzoate: 0.5, 1, 2, and 3 mg/mL; sodium saccharin: 0.5, 1, 1.5, and 2.5 mg/mL.

(g) *Spiked yogurt.*—0.5 mL stock solution from (f) was added to 'blank' yogurt to produce 5 g spiked yogurt, which was then carefully homogenized.

Extraction

A 0.5 g portion of thoroughly homogenized yogurt was transferred to a 30 mL glass screw-cap centrifuge tube; 7 mL phosphate buffer pH 5.5 was added, and the tube was ultrasonically mixed 2 min and then shaken 20 min in a mechanical shaker. After centrifugation (10 min at 2000 rpm), the supernatant liquid was transferred to a 25 mL volumetric flask. The extraction was repeated twice and the combined extracts were diluted to 25 mL with phosphate buffer pH 5.5 (extract A).

Five mL extract A was transferred to another centrifuge tube and shaken 20 min with 5 mL 0.01M TnOA solution in chloroform. After centrifugation (10 min at 2000 rpm), 2.5 mL organic phase was back-extracted with 2.5 mL 0.1M solution of sodium perchlorate in water. Phases were again separated by centrifugation.

Determination

Organic acids were determined as in ref. (5).

Results and Discussion

Separation of Benzoic Acid, Sorbic Acid, and Saccharin

Previous papers from our laboratory described the determination of synthetic dyes in various foodstuffs (2–4). We found that the ion-pair extraction with TnOA used for dyes can also be used for other substances, e.g., preservatives and saccharin. The LC separation of these compounds is best achieved with pH 4.5 mobile phases (5). All LC measurements described in this paper were carried out using the methanol–phosphate buffer (pH 4.5, ionic strength = 0.1) (40 + 60) mobile phase. Figure 1 shows a typical LC separation of food additives.

Extraction of Preservatives and Saccharin from Yogurt

Preliminary experiments showed that sorbate can be liberated from the yogurt matrix with phosphate buffer (pH 5.5) and sufficient methanol to produce a final methanol content of 40%. Because column clogging occurred under these conditions, direct chromatography proved to be impossible. Precipitation of the proteins did not help.

Eventually, the extraction scheme chosen consisted of the following 3 steps: first, an extraction with pH 5.5 phosphate buffer, second, an ion-pair extraction with TnOA, and, third, a back-extraction with perchlorate. As described before (5), recoveries are optimum using 0.01M TnOA and 0.1M sodium perchlorate. The advantages of this back-extraction were discussed in previous papers (2–4).

Detection was performed by wavelength programming because saccharin, which is very hydrophilic, co-elutes with some yogurt components. At the initial wavelength of 270 nm, absorbance of saccharin is maximal, and yogurt constituents show a lower absorption. After 4 min, the wavelength is automatically switched to 240 nm to increase (by a factor of 5) sensitivity to benzoic acid.

Table 1 gives extraction recoveries for sorbic acid, benzoic acid, and saccharin, spiked at different concentrations. Data
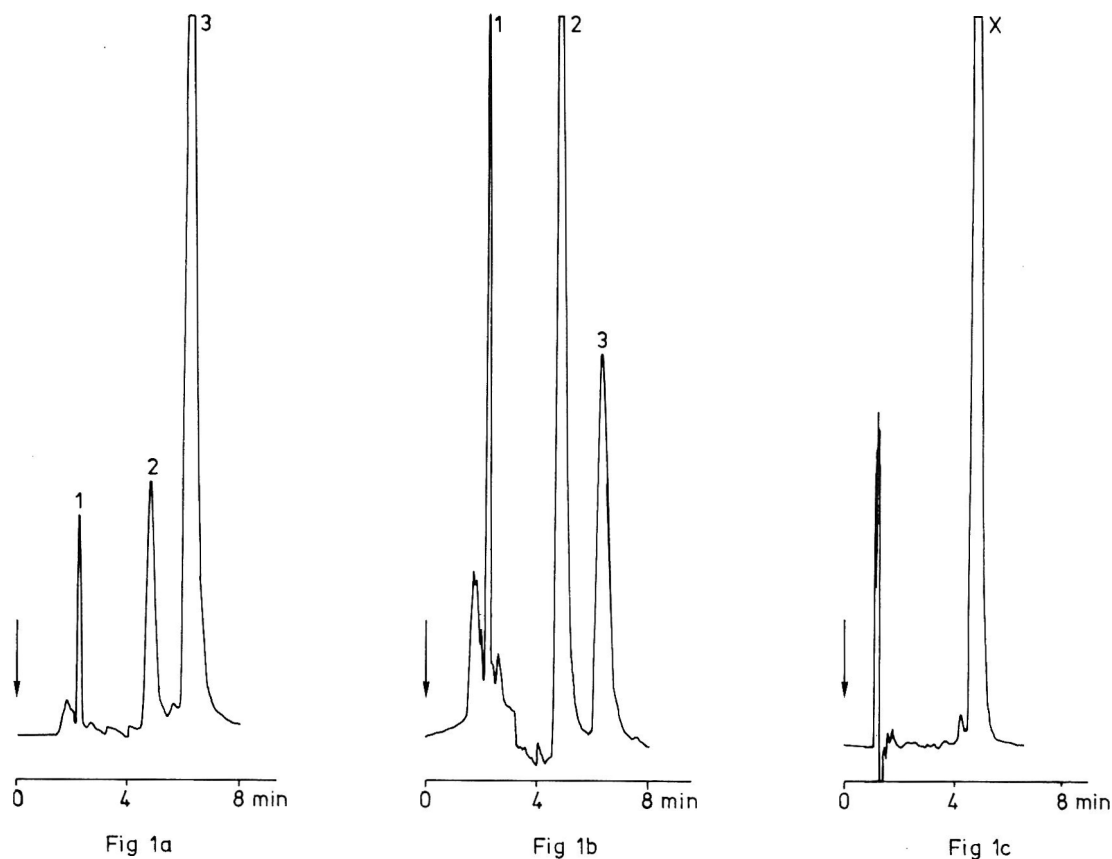
Figure 1. Separation of food additives: 1a, mixture of standards, 1 ppm of each in water: 1, sodium saccharin; 2, sodium benzoate; 3, potassium sorbate, 0.02 AUFS. 1b, Extract from yogurt spiked with 200 ppm sodium saccharin (1); 100 ppm sodium benzoate (2); and 20 ppm potassium sorbate (3), 0.02 AUFS. 1c, Extract from commercial yogurt sample: X= sorbate, 0.08 AUFS. For LC conditions, see *Results and Discussion*.

Table 1.   Recovery of additives from spiked yogurt samples

Additive, mg/kg	Recovery, %		
	Potassium sorbate	Sodium benzoate	Sodium saccharin
10	86.1 ± 0.8	—	—
50	88.3 ± 0.9	70.9 ± 2.2	88.3 ± 1.7
100	90.1 ± 1.2	72.7 ± 0.5	83.3 ± 1.9
150	—	—	84.1 ± 1.9
200	88.0 ± 1.3	74.2 ± 1.3	
250	—	—	82.3 ± 1.3
300	87.7 ± 1.6	73.8 ± 0.8	—

obtained for benzoic acid and sorbic acid indicate no significant effect of analyte concentration on recovery. For saccharin, however, recovery observed at 50 ppm is somewhat higher than at other levels. This positive error can be explained by interfering co-eluting yogurt constituents.

Mean extraction yields (about 88% for sorbate, 70% for benzoic acid, and 85% for saccharin) are similar to recoveries obtained from aqueous solutions (5) and were included in measuring additives in commercial samples. These yields also indicate that the yogurt matrix exerts no significant influence on the ion-pair extraction used. The precision of the method was determined at the 50 mg/kg level ($N = 5$). The following relative standard deviations were obtained: 1.1% for sorbate, 3.3% for benzoate, and 2.1% for saccharin.

With the extraction scheme given above, starting from 0.5 g of sample and with no concentration step, the detection limits are about 1 mg/kg for sorbate and 20 mg/kg for benzoate and saccharin. This difference results from differences between their specific absorption coefficients.

Analysis of Commercial Samples

Table 2 gives the sorbate contents of 28 yogurt samples with fruit or fruit aroma. We analyzed samples before and after August 1983 to check if manufacturers changed their production process. Some samples, i.e., those with a sorbate content much higher than 300 mg/kg, were controlled again in April 1983.

Data show that only samples with real fruit contain sorbate. Furthermore, it appears that the sorbate amount depends mostly on the manufacturer and not on the kind of fruit added. Indeed, all samples from manufacturer A contain a high amount of preservative for 2 samples, concentrations found were as high as 450 and 540 mg/kg. A few months later, it was observed that the manufacturer had already changed the production process, and the concentrations found were 300 and 340 mg/kg.

Only samples with a sorbate content higher than 150 mg/ kg were reanalyzed after August 1983. These samples were purchased in November 1983. Table 2 shows that most samples were below the 150 ppm limit. However, the pineapple sample from manufacturer A did not change between April and November, and the apricot sample still exceeded the 150 ppm limit.

With the analysis scheme used, no benzoic acid or saccharin was found in any sample analyzed.

Table 2. Potassium sorbate concentrations (mg/kg) found in commercial samples[a]

Manufacturer	Yogurt	Fruit	Oct. 1982–Feb. 1983	Nov. 1983
A	whole	strawberries	317.5 ± 7.2	122.0 ± 3.5
	skimmed	strawberries	298.3 ± 2.0	1.0 ± 0.0
	skimmed	forrest fruits	337.7 ± 2.6	11.6 ± 0.5
	skimmed	exotic fruits	283.5 ± 2.3	50.3 ± 0.4
	whole	exotic fruits	257.0 ± 4.2	49.8 ± 0.9
	skimmed	raspberries	290.4 ± 1.3	1.2 ± 0.1
	skimmed	kiwis	266.6 ± 4.5	—[b]
	whole	apricots	446.9 ± 1.4 (303.3 ± 5.4)[c]	166.0 ± 1.2
	whole	pineapple	541.7 ± 10.8 (342.6 ± 6.3)[c]	350.0 ± 8.3
	whole	lemon aroma	—	
	whole	vanillin aroma	—	
	whole	strawberry aroma	—	
	whole	apricot aroma	—	
B	skimmec	cherries	198.8 ± 3.5	117.0 ± 1.2
	skimmec	bilberries	180.5 ± 0.6	43.5 ± 1.9
	whole	apricots	163.2	61.1 ± 2.5
	whole	grenadine	2.3	
C	whole	strawberries	259.7	50.4 ± 1.3
D	skimmed	bananas	65.0	
E	skimmed	grenadine	—	
F	skimmed	grenadine	38.9 ± 0.1	
G	skimmed	kiwis	—	
H	whole	strawberry aroma	—	
	whole	strawberries	—	
	skimmed	strawberries	33.6 ± 1.8	
	skimmed	pineapple	19.9 ± 0.7	
	skimmed	unspecified mixture	29.6	
	skimmed	peaches + raspberries	14.8	

[a]Triplicate determinations, unless no standard deviation is given.
[b]Below detection limit (1 mg/kg).
[c]April 1983.

REFERENCES

(1) Belgian Food Legislation KB 22/7/1982
(2) Puttemans, M., Dryon, L., & Massart, D. L. (1982) *J. Assoc. Off. Anal. Chem.* **65**, 730–736
(3) Puttemans, M., Dryon, L., & Massart, D. L. (1982) *J. Assoc. Off. Anal. Chem.* **65**, 737–744
(4) Puttemans, M., Dryon, L., & Massart, D. L. (1983) *J. Assoc. Off. Anal. Chem.* **66**, 670–672
(5) Puttemans, M., Dryon, L., & Massart, D. L. (1984) *J. Assoc. Off. Anal. Chem.* **67**, 880–885

DRUGS

Simple Colorimetric Method for Determination of Thiamine Hydrochloride in Pharmauceuticals

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A simple colorimetric method is described for the determination of thiamine hydrochloride (vitamin B_1) in dosage forms. The method is based on measurement of a yellow complex formed when thiamine HCl is treated with *p*-methylaminophenol sulfate (Metol) under alkaline conditions. Compounds such as vitamins A, B_2, B_6, B_{12}, C, D, and E, and niacinamide, citric acid, liquid glucose, calcium pantothenate, biotin, liver extract, and folic acid do not interfere in the reaction. Extracting the complex into chloroform before quantitation enhances the stability of the reaction product and removes interference of water-soluble colored constituents in syrup samples. Statistical validation shows that the method is precise and accurate. Results agree well with those obtained by other methods in the literature.

Thiamine plays a fundamental role in intermediate carbohydrate metabolism in all living cells. The compound corrects and prevents nutritional diseases, and is essential to energy utilization by the cell.

The official compendia describe gravimetric (1, 2) and fluorometric (3) methods for determining thiamine hydrochloride (vitamin B_1) in pharmaceutical preparations. The gravimetric method is time consuming and cannot be applied to formulations that contain other nitrogenous bases. Measurement conditions are critical in the fluorometric method because thiochrome is gradually decomposed by UV light. Various colorimetric methods are also reported in the literature (4–10). Vitamin C interferes in most of the diazotization-based colorimetric procedures. The method recently reported by Sane et al. (10) requires use of a centrifuge for formulations that contain a high percentage of minerals, and reagent blank absorbance increases with time.

The simple colorimetric methods described here for the determination of thiamine HCl in various pharmaceutical formulations eliminate these interferences.

METHODS

Reagents

(a) *Thiamine HCl standard solution*—Dissolve 100 mg accurately weighed thiamine HCl in 100 mL water in volumetric flask. Dilute 10 mL of this solution to 100 mL with water.

(b) *Metol solution.*—Dissolve 100 mg Metol in 100 mL water.
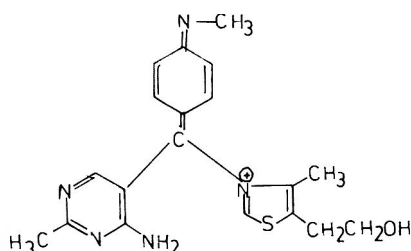
Figure 1. Proposed structure for product of reaction of thiamine and Metol.

Table 1. Formulations of products assayed

Content[a]	Amount	
Tablet A		
Each capsaliform tablet contains:		
Vitamin A acetate (IP)	25 000	IU
Vitamin D_3 (USP) (IP)	500	IU
Vitamin B_1 (IP)	20	mg
Vitamin B_6 (IP)	10	mg
Vitamin B_2 (IP)	5	mg
Vitamin B_{12} (IP)	5	μg
Niacinamide (IP)	50	mg
Calcium pantothenate (IP)	11.6	mg
Ascorbic acid (IP)	0.15	g
Vitamin E (NF)	10	mg
Biotin	0.25	mg
Minerals:		
Calcium phosphate (IP)	0.129	mg
Magnesium oxide, light (IP)	60.0	mg
Ferrous sulfate, dried (IP)	32.04	mg
Manganese sulfate (BPC)	2.03	mg
Phosphorus	25.8	mg
Copper sulfate (IP)	3.39	mg
Zinc sulfate (IP)	2.2	mg
Sodium molybdate	0.25	mg
Sodium borate (IP)	0.88	mg
Tablet B		
Each tablet contains:		
Vitamin B_1 (IP)	10	mg
Vitamin B_6 (IP)	3	mg
Vitamin B_{12} (IP)	33	mg
Tablet C		
Each tablet contains:		
Vitamin B_1 (IP)	2	mg
Vitamin B_6 (IP)	1	mg
Vitamin B_2 (IP)	11	mg
Niacinamide (IP)	20	mg
Calcium pantothenate (IP)	2.5	mg
Vitamin B_{12}	1	μg
Tablet D		
Each tablet contains:		
Vitamin B_1 (IP)	10	mg
Vitamin B_2 (IP)	10	mg
Vitamin B_6 (IP)	3	mg
Vitamin B_{12} (IP)	15	μg
Niacinamide (IP)	100	mg
Calcium pantothenate (IP)	50	mg
Folic acid (IP)	500	μg
Vitamin C	0.15	g
Syrup		
Each 5 mL contains:		
Vitamin B_1	2	mg
Vitamin B_2	2	mg
Niacin	5	mg
Niacinamide	15	mg
Vitamin B_6	1.5	mg
d-Panthanol	5	mg
Glucose, liquid	0.9	g
Citric Acid	35	mg
Plus syrup base		
Injection A		
Each mL contains:		
Vitamin B_1	100	mg
Chlorbutol (as preservative)	0.35	% v/v
Injection B		
Each mL contains		
Vitamin B_1	33	mg
Vitamin B_6	33	mg
Vitamin B_{12}	333	μg

[a] IP, Indian Pharmacopoeia; USP, U.S. Pharmacopoeia; NF, National Formulary; BPC, British Pharmacopoeia.

Table 2. Determination of thiamine HCl by the proposed method, for quantitation in aqueous and chloroform solutions

Product[a]	Amt declared, mg	Off. compendial method amt, mg(3)	Found, mg		Rec., %		Std dev.		Coeff. of var., %	
			Aq.	CHCl₃	Aq.	CHCl₃	Aq.	CHCl₃	Aq.	CHCl₃
Tablet										
A	20	22.39	24.4	22.24	101.8	101.3	0.25	0.26	1.8	3.2
B	10	12.47	12.54	11.9	102.06	100.08	0.24	0.22	2.6	2.7
C	2	2.87	2.6	2.9	100.2	101.2	0.175	0.25	1.89	2.8
D	10	13.14	13.7	12.6	99.99	103.10	0.20	0.27	2.05	2.8
Injection										
A	100	106.0	109	104.6	100.2	98.6	0.24	0.21	2.8	2.4
B	33	37.62	39	34.98	102.98	101.56	0.23	0.14	2.4	1.8
Syrup	2	2.49	2.51	2.44	104.4	100.86	0.24	0.20	2.6	2.1

[a]See Table 1 for product formulations.

Table 3. Recovery of thiamine hydrochloride added to formulations at 3 levels, for quantitation in aqueous and chloroform solutions

Product[a]	Amt declared, mg	Rec., %, in aq. soln			Rec., % in CHCl₃ soln		
		1	2	3	1	2	3
Tablet							
A	20	103.6	100.0	105.3	97.6	99.2	102.27
B	10	102.68	99.0	102.8	97.6	99.2	102.2
C	2	101.6	99.4	100.4	101.2	101.8	101.4
D	10	97.7	98.6	99.86	102.6	101.8	103.3
Injection							
A	100	97.6	102.8	100.4	98.8	98.6	98.8
B	33	101.6	104.2	102.0	99.2	104.8	101.6
Syrup	2	101.3	101.2	104.8	100.0	99.4	101.06

[a]See Table 1 for product formulations.

(c) *Liquor ammonia.*—AR grade.

(d) *Chloroform.*—AR grade.

Preparation of Assay Solutions

Tablets.—Powder 20 tablets and accurately weigh quantity equivalent to 10 mg thiamine HCl. Dissolve in ca 50 mL water, and filter solution through Whatman No. 1 paper. Collect filtrate and 2 washings each of 15 mL water in 100 mL standard flask and dilute to volume with water.

Injections.—From thoroughly shaken injection vial solution, transfer volume equivalent to 10 mg thiamine HCl to 100 mL standard flask, and dilute to volume with water.

Syrup.—From thoroughly shaken syrup sample, transfer volume equivalent to 10 mg thiamine HCl to 100 mL standard flask, and dilute to volume with water.

Preparation of Standard Curves

Dilute thiamine HCl standard solution to give series of concentrations of 2.5–20 µg/mL. Develop color and read absorbance of reaction product as described under *Assay.* Plot absorbance vs thiamine HCl concentration to give straight line passing through origin. Beer's law is obeyed between 2.5 and 20 µg/mL.

Assay

Method I.—Pipet 0.25–2.0 mL portions of thiamine HCl standard solution into series of 10 mL graduated test tubes. Add to each 0.5 mL Metol solution and 1.0 mL liquor ammonia. Simultaneously prepare reagent blank. Shake test tubes frequently for 7 min, dilute each solution to 10 mL, and measure absorbance at 420 nm against reagent blank. Determine concentration in assay solution from standard curve using same procedure detailed earlier.

Method II.—Pipet 0.25–2.0 mL portions of thiamine HCl standard solution into series of 10 mL graduated test tubes. Add 0.5 mL Metol solution and 1.0 mL aqueous ammonia to each solution. Simultaneously prepare reagent blank. Shake test tubes frequently for 7 min, and dilute each solution to 10

mL. Transfer contents of each test tube to a separate separatory funnel. To each funnel add 10 mL chloroform, and shake funnel 2 min. Let layers separate, and measure absorbance of chloroform layer at 420 nm against reagent blank. Determine concentration in assay solution from standard curve using same procedure detailed earlier.

Stoichiometry of Reaction Products

The yellow complex of thiamine with Metol was isolated. From the results of elemental analysis and UV and IR spectra of the compound, we propose the structure shown in Figure 1.

Recovery Experiment

To study the reliability and recovery of the proposed method, we used a method of standard addition. A fixed weight of sample was taken, standard drug was added at 3 different levels, and the total amount of drug was determined by the proposed method. Percent recovery was calculated as follows:

$$\text{Recovery, \%} = [N(\Sigma XY) - (\Sigma X)(\Sigma Y)]/[N(\Sigma X^2) - (\Sigma X)^2] \times 100$$

where X = amount of drug added/g sample; Y = amount of drug found/g sample; N = total number of observations.

Results and Discussion

The proposed methods are direct, fast, simple, sensitive, and accurate. Compounds such as vitamins A, B_2, B_6, B_{12}, C, D, and E, and niacinamide, biotin, liver extract, liquid glucose, calcium pantothenate, citric acid, and folic acid, do not interfere in the reaction.

Metol, an acid salt, is freely soluble in water and is stable in the powder form and in solution. The use of Metol in the present methods is an improvement over the use of *p*-ami-

nophenol, specified in the method of Sane and Ghorpade (11) for thiamine HCl. p-Aminophenol is susceptible to oxidation and, on standing, becomes dark brown in color; p-aminophenol solutions must be prepared in alcohol; and p-aminophenol is more expensive than Metol.

The stability of the Metol/thiamine colored complex is enhanced in the chloroform layer. For syrups, a sample blank must be analyzed because the colored constituents are water-soluble. This interference is eliminated by extracting the colored complex into chloroform. The results of analysis of thiamine HCl by the proposed methods agree well with those obtained by compendial methods.

Tablet, injection, and syrup dosage forms (Table 1) were assayed by the methods described, and recovery studies were carried out. Percent recoveries, obtained by using the additon method (Table 2), were in the range 99–103%, indicating lack of interference by other ingredients and excipients in the determination. The methods are also reproducible, as shown by results in Table 3. We recommend this method for the routine quality control analysis of thiamine HCl in pharmaceutical preparations.

REFERENCES

(1) *British Pharmacopoeia* (1980) Vol. I, Her Majesty's Stationery Office, London, UK, p. 451
(2) *Indian Pharmacopoeia* (1966) The Manager of Publications, Ministry of Health, Delhi, India, p. 511
(3) *U.S. Pharmacopoeia XX and National Formulary XV* (1980) U.S. Pharmacopeial Convention, Rockville, MD, p. 787
(4) Meinick, D., & Field, H. (1939) *J. Biol. Chem.* **127**, 505–514
(5) Watanabe, A., Kamio, H., & Tada, M. (1942) *J. Pharm. Soc. Jpn.* **62**, 1–4
(6) Kirch, E. R., & Dergeim, O. (1942) *J. Biol. Chem.* **143**, 575–588
(7) Sherman, O. S., & Kogan, S. M. (1953) *Trady Vsesoyuz N-I Vitaminogo In-ta* **4**, 230–234; (1954) *Chem. Abstr.* **49**, 9173i
(8) Haydene, K. J. (1957) *Analyst* **82**, 61–66
(9) Gupta, V. D., Cadwallander, D. E., Herman, H. B., & Honigberg, I. L. (1968) *J. Pharm. Sci.* **57**, 1109–1202
(10) Sane, R. T., Vaidya, U. M., & Deodhar, K. D. (1978) *Ind. J. Pharm. Sci.* **40**, 72–73
(11) Sane, R. T., & Ghorpade, U. A. (1983) *Acta Vitaminol. Enzymol.* **5**, 29–34

Liquid Chromatographic Determination of Primidone in Tablets: Collaborative Study

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Six laboratories collaboratively studied a liquid chromatographic (LC) method for the quantitative determination of primidone in tablets. Two lots each of commercially prepared 50 and 250 mg tablets and 2 authentic mixtures, at 50 and 250 mg levels, were sent to each collaborator. Samples were dissolved in the mobile phase, filtered, and injected into the chromatograph. Average recoveries for the 8 samples ranged from 97.5 to 101.2%, and coefficients of variation ranged from 0.53 to 3.01%. The LC method has been adopted interim official first action.

Primidone is a widely used anticonvulsant drug; its determination in solid dosage forms has been of international interest. At the time of this study, the USP method (1) for determining primidone in tablets was a nonspecific ultraviolet (UV) spectrophotometric procedure that is subject to interferences from other UV-absorbing materials. (By the time this study was completed, USP had adopted a new GC method (2).) A liquid chromatographic (LC) method was developed and evaluated by an interlaboratory collaborative study.

Collaborative Study

Eight samples were sent to each of 6 collaborators. Four samples were ground commercial tablets, 2 each labeled to contain 50 and 250 mg primidone, and 4 were authentic mixtures, 2 each at the 50 and 250 mg levels. The authentic mixtures were prepared to represent the most common formulations manufactured. The 8 samples were actually 4 samples in blind duplicate. A primidone standard was sent to each collaborator so that all samples and standards were identical for each set.

Equipment, reagents, and the LC procedure have been previously described (3). The collaborators were instructed to perform one determination on each sample and to report their LC results based on peak height.

Primidone in Tablets
Liquid Chromatographic Method
Interim First Action

Principle

Sample is dissolved in mobile phase, filtered, injected into liq. chromatge system, and quantitated by comparison with external std.

Reagents

(a) *Solvents.*—LC grade H_2O and MeOH (J. T. Baker Chemical Co., Phillipsburg, NJ 08865).

(b) *LC mobile phase.*—In suitable flask, combine 500 mL H_2O and 500 mL MeOH; stir mag. Filter thru 0.45 μm membrane filter wetted with MeOH. Place in ultrasonic bath 10 min to deaerate.

(c) *Std soln.*—Accurately weigh 50 mg USP Ref. Std Primidone, previously dried 2 h at 105°, and transfer to 50 mL vol. flask. Add 35 mL mobile phase, place in ultrasonic bath 15 min, cool, and dil. to vol. with mobile phase. Place in ultrasonic bath for addnl 15 min and cool. Soln is stable 1 week.

Table 1. Collaborative results of LC determination of primidone in commercial tablets and authentic mixtures[a]

Coll.	A mg	A %	B mg	B %	C mg	C %	D mg	D %	E mg	E %	F mg	F %	G mg	G %	H mg	H %
1	50.1	100.2	48.9	97.8	246.8	98.7	241.7	96.7	48.1	96.2	50.4	100.8	231.3	92.6	252.6	101.0
2	49.7	99.3	50.8	101.7	248.5	99.4	247.7	99.1	48.5	97.0	49.3	98.7	238.7	95.5	251.7	100.7
3	50.5	101.0	50.8	101.6	249.3	99.7	243.7	97.5	49.9	99.8	50.0	100.0	251.6	100.6	243.2	97.3
4	50.3	100.6	50.1	100.2	245.8	98.3	240.9	96.4	50.3	100.6	50.0	100.0	246.8	98.7	246.2	98.5
5	50.5	101.1	51.3	102.5	246.7	98.7	246.3	98.5	50.0	100.0	49.5	98.9	247.6	99.0	247.8	99.1
6	50.0	100.0	51.7	103.4	248.1	99.2	247.0	98.8	48.6	97.2	49.4	98.8	247.1	98.8	248.9	99.6
mg/tab., declared	50		50		250		250		50		50		250		250	
Av. wt/tab., mg	114.3		114.3		377.1		377.4		114.6		114.6		276.6		276.6	
Av., mean	50.2	100.4	50.6	101.2	247.5	99.0	244.6	97.8	49.2	98.5	49.8	99.5	243.9	97.5	248.4	99.4
SD	0.31	0.68	0.99	1.97	1.31	0.52	2.87	1.13	0.94	1.87	0.43	0.86	7.45	2.93	3.49	1.39
CV, %	0.62	0.68	1.96	1.95	0.53	0.53	1.17	1.16	1.91	1.90	0.87	0.86	3.05	3.01	1.41	1.40

[a] Samples A–D were commercial tablets; Samples E–H were authentic mixtures. A and B, C and D, E and F, and G and H were duplicate pairs, respectively.

Apparatus

(a) *Liquid chromatograph.*—System equipped with injector, solvent delivery system, and UV detector. Operating conditions: flow rate 1.0 mL/min; 254 nm detector, 0.2 AUFS; temp., ambient; 20 μL injection.

(b) *LC column.*—Macherey-Nagel Nucleosil C-8, 10 μm particle size, 25 cm × 3.2 mm, or equiv.

(c) *Recorder.*—10 mV with 0.5 cm/min chart speed.

(d) *Membrane filters.*—Nylon-66, pore size 0.45 μm (Rainin Instrument Co., Woburn, MA 01890), or equiv.

Preparation of Samples

Det. av. wt of 20 tablets and grind to pass No. 60 sieve. Transfer accurately weighed portion of powder equiv. to 50 mg primidone to 50 mL vol. flask. Add 35 mL mobile phase, place in ultrasonic bath 15 min, cool, and dil. to vol. with mobile phase. Place in ultrasonic bath addnl 15 min and cool. Filter soln through 0.45 μm membrane filter and use as sample prepn.

Determination

Equilibrate system with column in instrument and mobile phase set at 1.0 mL/min. Inject 20 μL std soln and adjust flow rate and sensitivity so that peak response is ca 45% full scale, with retention time ca 3 min. In suitable system, coefficient of variation (CV) of peak responses of 5 replicate injections is ≤2.0%. Proceed with sample analysis, using 20 μL injections for each std and sample soln.

Calculation

Det. peak responses of std and sample peaks and calc. amt of primidone in tablets.

$$\text{mg Primidone/tablet} = (R/R') \times 50 \, (C) \, (T)/W$$

where R and R' are peak response of sample and std solns, resp.; C is concn of primidone std soln in mg/mL; T is av. tablet wt in mg; and W is sample wt in mg.

Results and Discussion

Each collaborator was instructed to analyze the samples in any order, but only once for each sample; however, some collaborators used duplicate injections, some boxed each sample with a standard injection, and others did both. All collaborators used peak height measurements as requested in the instructions; however, the method has been changed to measurement by peak response to allow the choice of area or height. Table 1 shows the results of the collaborative study.

None of the collaborators had problems with the method; however, one collaborator recommended using 3 or more injections for each sample or using an internal standard, which would produce more accurate results statistically. Multiple injections, at least 2, would be more accurate; some collaborators did this. The system suitability test, however, requiring 5 replicate injections to meet a relative standard deviation (RSD) of 2.0% or less was met by all collaborators, which indicates that there are no problems associated with injection or stability. All collaborators except one achieved an RSD of <0.9%, with a range of 0.17 to 1.88% for all 6. Peak symmetry was satisfactory, the maximum being about 1.2, and no interferences were noted in any of the chromatograms received.

Table 2 lists the equipment that the collaborators used. Five collaborators used C-8 columns from various manufacturers, as specified in the method, and one used a phenyl column because of unavailability of a C-8 column. The use of the phenyl column did not appear to affect the results obtained, and the data were included in the statistical evaluation. All other equipment was expectedly variable.

Statistical Analysis

Accuracy.—The data from the 6 collaborating laboratories (Table 1) were statistically analyzed. The mean and SD for Collaborators 1–6 were 98.0 (2.84), 98.9 (1.95), 99.7 (1.55), 99.2 (1.46), 99.7 (1.40), and 99.5 (1.78). The means fell within a narrow range, but the SDs showed a wider spread. The authentic mixtures had a significantly higher SD than the commerical tablets. The average SD for the commercial tablets was 1.08 and for the authentic mixtures was 1.76. This lack of precision is probably ascribable to the difficulty of preparing homogenous authentic mixtures on a small scale.

Homogeneity of variance.—Before an analysis of variance (ANOVA) was performed, the homogeneity of variance was examined. When the complete data set consisting of 50 and 250 mg samples was pooled, the variance was found to be sufficiently homogeneous at the 99% confidence level. This was true for both the Hartley and the Cochran tests. For this reason no laboratory was eliminated, and the ANOVA was performed on all of the collaborative results.

Analysis of variance.—The analysis of variance on the complete set of data (Table 3) showed that the between-collaborator effect and the laboratory-sample interaction were insignificant at the 95% confidence level. In fact, the largest amount of scatter in the data, by far, can be attributed to the between-replicate variability. As noted above, this is primar-

Table 2.   LC equipment used by collaborators

Coll.	Instrument	Injector	Column	Detector[a]
1	Waters 204	Waters U6K	Nucleosil C-8	Waters 440
2	Altex 101	Waters WISP 710A Autosampler	μBondapak phenyl (slurry packed) (commercial)	Perkin-Elmer LC-75
3	Haskel	20 μL loop	Hewlett-Packard RP-8 (commercial)	LDC Spectromonitor III
4	Waters 6000A	Waters U6K	Waters Radial Pak C-8 (commercial)	Waters fixed
5	Varian 5060	Micromeritics 725 Autosampler	DuPont Zorbax C-8 (commercial)	LDC Spectromonitor III
6	Altex 100A	20 μL loop	DuPont Zorbax C-8 (commercial)	LDC Spectromonitor III

[a]All detectors were set at 254 nm.

Table 3.   Analysis of variance

Factor	Degrees of freedom	Sum of squares	Mean square (MS)	F-value
Between-laboratories (L)	5	16.84	3.37	2.73[a]
Between samples (S)	3	44.46	14.82	
Laboratory-sample interaction (LS)	15	18.51	1.23	0.337[b]
Between-replicates (0)	24	87.98	3.66	
Total	47	167.79		

[a]$F = MS_L/MS_{LS}$, critical F (5/15 degrees of freedom) $= 2.9$ at 95% significance level.

[b]$F = MS_{LS}/MS_0$, critical F (15/24 degrees of freedom) $= 2.0$ at 95% significance level.

ily caused by the authentic mixture samples, which apparently were not homogeneous.

Repeatability and reproducibility.—From the ANOVA table, the variance of each type in the analysis was calculated. The square root of the between-replicate variance, 1.91, corresponds to the repeatability of the method. The between-laboratory SD was 0.517 and the laboratory sample interaction was <0. The square root of the sum of the between-laboratories, between-replicates, and laboratory-sample variances corresponds to the overall reproducibility of the method, 1.94, which is only slightly larger than the repeatability, for reasons discussed above. The 95% confidence limit of the method, taken as twice the reproducibility, corresponds to a limit for the analytical method of ±4%.

An ANOVA test was performed on the commercial and authentic samples separately. The reproducibility and repeatability results were 2.45 and 2.39 for the authentic mixtures and 1.44 and 1.26 for the commercial tablets, respectively. The excellent agreement between the measured and declared potency of each sample, however, demonstrates the accuracy of the LC method.

Recommendation

It is recommended that the LC method for the determination of primidone in tablets be adopted official first action.

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REFERENCES

(1) *United States Pharmacopeia* (1980) 20th Rev., U.S. Pharmacopeial Convention, Inc., Rockville, MD, p. 658

(2) *United States Pharmacopeia* (1980) 20th Rev., U.S. Pharmacopeial Convention, Inc., Rockville, MD, Addendum A to 4th Supplement (1983), p. 879

(3) Roberts, S. E. (1982) *J. Assoc. Off. Anal. Chem.* **65**, 1063–1065

Normal Phase Liquid Chromatographic Determination of Sulfamethoxazole in Tablets: Collaborative Study

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A stability-indicating liquid chromatographic method is presented for determining sulfamethoxazole in tablets. The method uses a 10 μm silica column, an isooctane–methylene chloride–2-propanol–acetonitrile–glacial acetic acid (70 + 25 + 5 + 5 + 0.5) mobile phase, and photometric detection at 254 nm. Seven laboratories collaboratively studied this method on powdered composite samples prepared from commercial 500 and 1000 mg tablets and on an authentic tablet mixture containing 83.32% added sulfamethoxazole. Mean assay results for the 500 and 1000 mg tablets were 102.2 and 97.9% of declared, respectively ($n = 4$). The mean recovery value for the synthetic sample was 99.4% ($n = 4$). The pooled reproducibility standard deviation (SD) (coefficient of variation (CV)) and pooled repeatability SD (CV) were ± 1.01 (1.01%) and ± 0.96 (0.96%), respectively. These results were in good agreement with those obtained by the Associate Referee for the titration method of USP XX. The proposed method can also be used for monitoring the presence of sulfanilamide in sulfamethoxazole by increasing the proportions of both acetonitrile and 2-propanol in the mobile phase. The method has been adopted official first action.

Several analytical methods have been reported for determination of the antibacterial, sulfamethoxazole, the most common being the USP XX titrimetric method with nitrite solution (1). This method is precise but nonspecific, and many drugs, including most of the sulfonamides, react with the titrant. Quantitative thin layer chromatography (TLC) (2–5), which separates sulfamethoxazole from related compounds and excipients, lacks the accuracy and precision needed for both quality control and regulatory purposes. These necessary analytical criteria can be met by using gas chromatographic (GC) methods (6–8), but these procedures require the derivatization of sulfamethoxazole, which is usually a time-consuming and more error-prone step. Liquid chromatography (LC) is preferred over TLC because of its greater precision and resolution power, and over GC because derivatization is unnecessary.

The LC method proposed here is an outgrowth of a need in our laboratory to analyze sulfamethoxazole for impurities. A number of reverse phase LC methods for this drug have been published (9–14). However, with reverse phase systems, sulfamethoxazole shows a retention time in excess of 1 h, whereas impurities such as sulfanilic acid elute close to the solvent front. The normal phase LC method of Cobb and Hill (15), on the other hand, appeared more promising and was used as the basis for the development of the present method.

Collaborative Study

Three samples, consisting of commercial 500 and 1000 mg sulfamethoxazole tablets and an authentic sulfamethoxazole tablet mixture, were submitted to 8 collaborators as blind duplicates, for a total of 6 samples. Each collaborator was asked to analyze all of these samples in duplicate. Each collaborator also received samples of standard sulfamerazine

and sulfamethoxazole and a set of instructions for carrying out the study.

The authentic tablet mixture was prepared in 2 steps. The ingredients, in the amounts given in Table 1, were mechanically dry mixed 2 h in a 1 L Morton flask. A 33.36 g portion of this mixture, 166.64 g sulfamethoxazole, and 400 mL methanol were combined in a 1 L Morton flask and mechanically rotated under vacuum for 12 h. The methanol was evaporated on a steam bath. The residue was finely powdered (60 mesh) and dried 2 h at 105°C.

Sulfamethoxazole in Tablets
Liquid Chromatographic Method
First Action

Principle

Sulfamethoxazole is extd with MeOH, sulfamerazine is added as internal std, and compds are detd by liq. chromatgy on normal phase silica column with isooctane–CH_2Cl_2–2-propanol–CH_3CN–HOAc (70 + 25 + 5 + 5 + 0.5) mobile phase and UV detector set at 254 nm.

Apparatus and Reagents

(a) *Liquid chromatograph.*—Isocratic system operated at room temp., with UV detector set at 254 nm, and strip chart recorder or computing integrator.

(b) *Analytical column.*—Stainless steel, 250 × 4.6 (id) mm, packed with 5–10 μm particle size, uncoated silica (μPorasil, Waters Associates, or equiv.). At mobile phase flow rate of 2 mL/min, approx. retention times of sulfamethoxazole and sulfamerazine are 3 and 4 min, resp.

(c) *Mobile phase.*—Isooctane–CH_2Cl_2–2-propanol–CH_3CN–HOAc (70 + 25 + 5 + 5 + 0.5).

(d) *Internal std soln.*—Dissolve USP Sulfamerazine Ref. Std in MeOH in vol. flask to give soln contg ca 2.0 mg/mL.

(e) *Sulfamethoxazole std soln.*—Dissolve accurately weighed amt of USP Sulfamethoxazole Ref. Std in MeOH in vol. flask to obtain soln contg ca 5.0 mg/mL.

Preparation of Sample Solution

Weigh and finely powder ≥ 20 tablets. Accurately weigh portion of powder equiv. to ca 500 mg sulfamethoxazole and transfer to 100 mL vol. flask. Dissolve in 70 mL hot MeOH. Cool soln to room temp., dil. to vol. with MeOH, mix, and filter.

Table 1. Composition of authentic tablet excipient

Ingredient	Amount, mg
Avicel A 80[a]	5.5
Primojel[c]	14.4
Explotab[b]	12.0
St-Rx[c]	10.6
Pre-Jel[c]	7.6
Stearic acid	40.0
Sodium lauryl sulfate	20.0
Lactose	50.0

[a]Microcrystalline cellulose.
[b]Modified starch.
[c]Pregelatinized starch.

Table 2. Collaborative results (% of declared) for the LC determination of sulfamethoxazole in commercial tablets and authentic tablet mixture

| Coll. | Commercial tablet | | | | | | | | Authentic tablet mixture[a] | | | |
	500 mg				1000 mg							
A	100.7	101.4	100.4	100.4	94.5	94.2	98.9	98.9	98.5	99.7	99.0	99.1
B	102.4	103.2	102.4	101.9	97.1	99.2	98.6	97.5	99.3	99.9	99.6	97.6
C	101.8	101.6	101.3	102.2	97.3	98.5	98.1	98.8	100.2	100.0	99.3	100.5
D	102.0	102.2	102.2	101.8	98.2	98.1	98.1	98.2	99.3	99.7	98.5	100.5
E	101.5	102.3	104.9	102.5	101.7[b]	100.7[b]	99.8[b]	101.4[b]	100.0[c]	93.5[c]	100.6[c]	100.5[c]
F	102.4	102.7	102.6	102.5	99.2	98.0	97.7	97.8	99.6	100.0	98.5	99.5
G	102.6	102.7	101.1	103.5	97.7	98.3	100.3	97.4	101.9[b]	101.7[b]	101.4[b]	102.2[b]
Mean, %				102.1				97.9				99.4
Pooled mean, %				99.8								
Reproducibility SD, % (CV, %)				± 1.01 (1.01)								
Repeatability SD, % (CV, %)				± 0.96 (0.96)								

[a]Containing 83.32% added sulfamethoxazole.
[b]Outlier by Dixon test.
[c]Outlier by range test.

Table 3. Evaluation of collaborative results for outliers by Steiner's ranking test[a]

| Coll. | Commercial tablets | | | | Authentic tablet mixture | | Overall rank |
| | 500 mg | | 1000 mg | | | | |
	Sum	Rank	Sum	Rank	Sum	Rank	
A	422.9	1	386.5	1	396.3	2	4
B	409.9	4.5	392.4	2	396.4	3	9.5
C	406.9	2	392.7	4.5	400.0	6	12.5
D	408.2	3	392.6	3	398.0	5	11
E	411.2	7	403.6	7	394.6	1	15
F	410.2	6	392.7	4.5	397.6	4	14.5
G	409.9	4.5	393.7	6	407.2	7	17.5

[a]P = 0.05; for 7 collaborators and 3 samples, degrees of freedom (df) = 21.
Conclusion: no outliers found.

Table 4. Evaluation of outliers between samples by the Dixon test[a]

Sample	Highest value, [b] r_{10}	Lowest value, [c] r_{10}
Tablet, 500 mg	0.12	0.48
Tablet, 1000 mg	0.58	0.34
Authentic tablet mixture	0.57	0.13

[a]Critical value (P = 0.05) for 7 collaborators (r_{10}) = 0.51.
Conclusion: 2 outliers found.
[b]For highest value use $(X_n - X_{n-1})/(X_n - X_1)$; reject if r_{10} >0.51.
[c]For lowest value use $(X_2 - X_1)/(X_n - X_1)$; reject if r_{10} >0.51.

Table 5. Evaluation of outliers between replicates by the range test[a]

| Coll. | Commercial tablet diff. | | Authentic mixt. diff. |
	500 mg tablet	1000 mg tablet	
A	0.1	4.7	1.2
B	1.3	2.1	2.3
C	0.9	1.5	1.2
D	0.4	0.1	2.0
E	3.4	—[b]	7.1
F	0.3	1.5	1.5
G	2.4	2.9	—[b]

[a]Total difference: 36.9. Critical value (P = 0.05) = 0.13 when number of ranges is 19 and number of values in each range is 4.
Ratio of greatest difference (7.1) = 0.19.
Conclusion: 1 outlier found.
Ratio of next greatest difference (4.7) = 0.12.
Conclusion: 1 outlier found.
[b]Outlier by Dixon test.

Determination

Transfer 2.0 mL each of sample soln and std soln to individual 100 mL vol. flasks contg 5.0 mL internal std soln, add 20 mL CH_2Cl_2, dil. to vol. with mobile phase, and mix.

Using suitable micro syringe or sampling valve, test system suitability by making 5 replicate injections (between 10 and 20 μL) of std mixt. If necessary, adjust injection vol. and flow rate to give std peak ht of ca 60% AUFS. In properly functioning system. resolution factor between std and internal std peaks is ≥ 2.5, and CV for 5 replicate injections is ≤ 3.0% for peak ht ratios of std to internal std.

Introduce equal vols (between 10 and 20 μL) of sample soln and std soln into liq. chromatograph operated at room temp. For each injection, calc. response ratio (ht of sulfamethoxazole peak to ht of internal std peak).

Sulfamethoxazole, mg/tab. = $100\ C \times (R/R') \times (T/W)$

where C = concn, mg/mL, of USP Sulfamethoxazole Ref. Std in std soln; R and R' = ratios for sample and std solns, resp.; T = av. tablet wt, g; and W = sample wt, g.

Results and Discussion

Analytical findings were received from 7 of the 8 collaborators. The commercial 500 and 1000 mg tablet samples showed mean assay values of 102 and 98% of declared, respectively. The authentic tablet mixture, containing 83.32% sulfamethoxazole, had a mean value of 99.4% of expected. The pooled mean for the 3 samples was 99.8% (Table 2).

With Steiner's ranking test (P = 0.05), no outliers were found among the results submitted by the collaborators (Tables 2 and 3). However, 2 sets of results were found as outliers by the Dixon test (Collaborators E and G, Tables 2 and 4) and 1 set by the range test (Collaborator E, Tables 2 and 5). These results were not included in the statistical evaluations (16).

When the results from the collaborators for the 3 samples analyzed were pooled (Table 2), the reproducibility standard deviation (SD) was ± 1.01 and the reproducibility coefficient of variation (CV) was 1.01%. The corresponding repeatability SD and the repeatability CV were ± 0.96 and 0.96%, respectively.

Table 6.   Analysis of variance (ANOVA) of collaborative results

Source of variance	Sum of squares	df	Mean square	F-statistic[a]
Between collaborators	14.0	6	2.30	3.77
Between samples	240.8	2	120.40	—
Laboratory/sample interaction	7.4	12	0.61	0.74
Between replicates	49.2	60	0.82	—

[a]$F(0.05)$ [6, 12] = 3.00; $F(0.05)$ [12, 60] = 1.92 F (0.01) [6, 12] = 4.82.

Table 7.   LC equipment used by collaborators

Coll.	Column	System Pump	System Detector	System Integrator
A	Spherisorb S-5-W[a]	M-6000[b]	LC 75[c]	3390A[d]
B	Zorbax-Sil[e]	SP 3500[f]	SP 8200[f]	—[g]
C	μPorasil[b]	202[b]	401[b]	—[g]
D	Zorbax-Sil[e]	204[b]	440[b]	—[g]
E	μPorasil[b]	950[h]	970[h]	SP 4100[f]
F	LiChrosorb Si-60[i]	110A[i]	334[i]	3385A[e]
G	μPorasil[b]	SP 8000[b]	SP 8000[b]	SP 8000[b]

[a]Alltech Associates, Inc., Deerfield, IL.
[b]Waters Associates, Milford, MA.
[c]Perkin-Elmer Corp., Norwalk, CT.
[d]Hewlett-Packard Co., Palo Alto, CA.
[e]DuPont Instruments, Wilmington, DE.
[f]Spectra-Physics, Inc., Mountain View, CA.
[g]Information not available.
[h]Tracor, Inc., Austin, TX.
[i]Altex Scientific Inc., Berkeley, CA.

Table 8.   Associate Referee's analytical results by proposed and USP XX methods

Sample	Method LC	Method USP XX
Tablet, 500 mg	101.9	102.8
Tablet, 1000 mg	100.1	98.9
Synthetic tablet, 83.32% sulfamethoxazole	100.4	100.1

The analysis of variance (ANOVA) test (Table 6) shows that the F-statistic between collaborators was significant at the 95% confidence level, whereas the laboratory/sample interaction F-statistic was not. Usually the variance between collaborators is much greater than the variance due to within-laboratory/sample interaction. Therefore, it is very unusual to find that the precision among laboratories was almost the same as the precision within laboratories.

Overall, these results show that the accuracy and the precision of the proposed method are satisfactory regardless of the variation in the chromatographic system used (Table 7). Close agreement between the results obtained by the proposed and USP XX methods was obtained by the Associate Referee for the same samples that were sent to each collaborating laboratory (Table 8).

The relationship between the response of the detector and the amount of sulfamethoxazole injected was linear over the range 0.0–3.0 μg injected drug. Figure 1 shows a typical chromatogram of a mixture of sulfamethoxazole and the internal standard sulfamerazine.

This method will also resolve a mixture of sulfamethoxazole with its acid hydrolysis degradation product sulfanilamide if the volumes of 2-propanol and acetonitrile in the mobile phase are increased by about 50%, i.e., an increase from 5 parts to 7.5 or 8 parts for each. Under these conditions, sulfanilamide elutes with a retention time of approximately
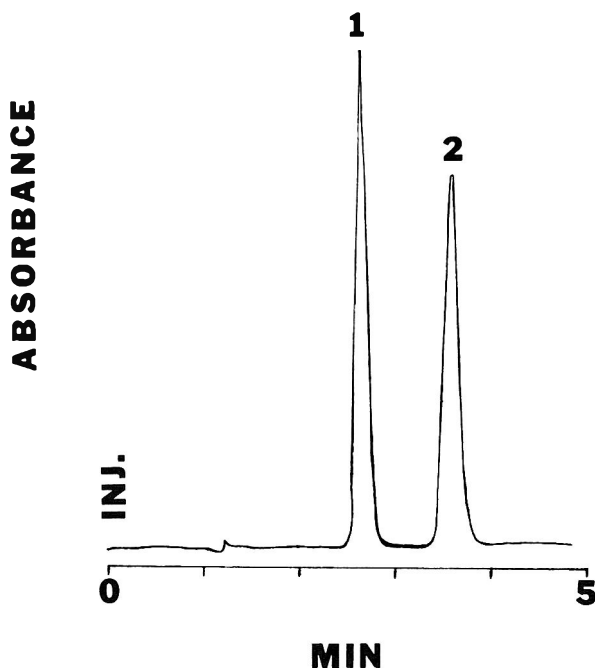
Figure 1.   LC separation of 1, sulfamethoxazole, and 2, sulfamerazine. Chromatographic conditions: column, Zorbax-Sil (USP XX, Type L3); mobile phase, isooctane–methylene chloride–2-propanol–acetonitrile–glacial acetic acid (70 + 25 + 5 + 5 + 0.5); flow rate, 2.0 mL/min; detector, 254 nm; sensitivity, 0.32 AUFS.

20 min with some loss in resolution for the separation of the parent compound and the internal standard. In contrast, when the original mobile phase is used, sulfanilamide elutes more slowly and appears in the chromatogram as a broad, shallow, tailing peak.

Recommendation

It is recommended that the LC determination of sulfamethoxazole in tablets be adopted official first action.

Acknowledgments

REFERENCES

(1) *U.S. Pharmacopeia* (1980) 20th Rev., U.S. Pharmacopeial Convention, Rockville, MD, p. 749

(2) Sigel, C. W., Woolley, J. L., Jr, & Nichol, C. A. (1975) *J. Pharm. Sci.* **64**, 973–976

(3) Sigel, C. W., Grace, M. E., Nichol, C. A., & Hitchings, G. H. (1974) *J. Pharm. Sci.* **63**, 1202–1205

(4) Biagi, G. L., Barbaro, A. M., Guerra, M. C., Cantelli-Forti, G., & Gandolfi, O. (1975) *J. Chromatogr.* **106**, 349–355

(5) De Silva, J. A. F., & Strojny, N. (1975) *Anal. Chem.* **47**, 714–718

(6) Dadisch, G. L., Vycudilik, W., & Machata, G. (1977) *Forensic Sci.* **10**, 205–216

(7) Nose, N., Kobayashi, S., Hirose, A., & Watanabe, A. (1976) *J. Chromatogr.* **123**, 167–173

(8) Vree, T. B., Hekster, Y. A., Baars, A. M., Damsma, J. E., & van der Kleijn, E. (1978) *J. Chromatogr.* **146**, 103–112

(9) Johansson, I. M., & Wahlund, K. -G. (1977) *Acta Pharm. Suec.* **14**, 459–474

(10) Bye, A., & Brown, M. E. (1977) *J. Chromatogr. Sci.* **15**, 365–371

(11) Helboe, P., & Thomsen, M. (1977) *Arch. Pharm. Chemi Sci. Ed.* **5**, 435–460; through (1977) *Chem. Abstr.* **87**, 90800j

(12) Butterfield, A. G., Matsui, F. F., Smith, S. J., & Sears, R. W. (1977) *J. Pharm. Sci.* **66**, 684–687

(13) Helboe, P., & Thomsen, M. (1977) *Arch. Pharm. Chemi Sci. Ed.* **5**, 25–32; through (1977) *Int. Pharm. Abstr.* **14**, 4850

(14) Henry, D., Block, J. H., Anderson, J. L., & Carlson, G. R. (1976) *J. Mea. Chem.* **19**, 619–626

(15) Cobb, P. H., & Hill, G. T. (1976) *J. Chromatogr.* **123**, 444–447

(16) Youden, W. J., & Steiner, E. H. (1975) *Statistical Manual of the AOAC*, AOAC, Arlington, VA

Colorimetric Determination of Sympathomimetic Amines Methyldopa and Noradrenaline

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The chromogenic reagent *p*-dimethylaminocinnamaldehyde (PDAC) is introduced for the determination of the sympathomimetic amines methyldopa and noradrenaline. The method is based on measurement of the orange color developed when the alkaline solution of methyldopa and noradrenaline is allowed to react with PDAC at pH 5.0. The color developed obeys Beer's law in the concentration range 0.1–1.5 mL of 2×10^{-3}M solution of noradrenaline and methyldopa. The results are compared with those obtained with another chromogenic reagent, *p*-dimethylaminobenzaldehyde (PDAB). Determinations on dosage forms of the drugs, using PDAC and PDAB reagents, agreed well with results of determinations by official pharmacopoeial methods.

Several procedures for the determination of sympathomimetic amines have been described, including gas and liquid chromatography (1–4), fluorescence and phosphorescence (5), thin layer chromatography combined with fluorometric techniques (6), and titrimetric and spectrophotometric procedures (7, 8).

The purpose of our investigation was (a) to develop a simple, rapid, more convenient method for the estimation of some sympathomimetic amines, especially noradrenaline, in the presence of adrenaline without previous extraction, and also for methyldopa estimation; and (b) to apply the procedure to pharmaceutical formulations on the Egyptian market.

The method that we developed is a modification of the procedure of Shelton and Henry (9). Methyldopa and noradrenaline are estimated by treating the alkaline solutions with *p*-dimethylaminocinnamaldehyde (PDAC) in sodium acetate medium and measuring the absorbance of the resulting orange complex at 440 nm. In addition, we used the procedure of Ramana and Krishnan Sivarama (10) for colorimetric estimation of methyldopa, using *p*-dimethylaminobenzaldehyde (PDAB). We substituted sodium acetate reagent for the pH 1.25 glycine buffer specified in their method. Figure 1 shows typical absorption spectra of the colored complexes developed following reaction of the drugs within the 2 reagents.

Experimental

Apparatus and Reagents

All chemicals were analytical reagents from E. Merck AG.

(a) *Spectrophotometer.*—Perkin Elmer 550S.

(b) *p-Dimethylaminocinnamaldehyde.*—(Aldrich Chemical Co.) 0.1% w/v in methanol.

(c) *p-Dimethylaminobenzaldehyde.*—(Hopkin and William Ltd.) 1% w/v in methanol.

(d) *Sodium hydroxide.*—1.0% w/v in methanol.

(e) *Sodium acetate.*—50% in methanol.

(f) *L-Noradrenaline, L-adrenaline, and methyldopa.*—(BDH Chemicals, Ltd.) Prepare 2×10^{-3}M solutions by dissolving 0.34, 0.367, and 0.48 g of each separately in 1 L volumes of 0.01M acetic acid in methanol.

Procedure A—Pure Drugs, Using PDAC Reagent

Into 10 mL volumetric flask, introduce accurately measured volume of 0.01M acetic acid in methanol that contains up to 500 µg noradrenaline or methyldopa. Add 0.5 mL alcoholic sodium hydroxide, and let solution stand 5 min. Then add 1.0 mL sodium acetate solution followed by 0.5 mL PDAC reagent. Dilute solution to volume with methanol, wait 15 min, and then determine absorbance at 440 nm against reagent blank similarly prepared. Read concentration or noradrenaline or methyldopa from calibration curve prepared in same manner, using concentrations ranging from 30 to 500 µg noradrenaline and from 40 to 600 µg methyldopa.

Procedure B—Pure Drugs, Using PDAB Reagent

Follow Procedure A, except add 1.0 mL PDAB reagent instead of 0.5 mL PDAC reagent, and measure absorbance at 420 nm (noradrenaline) or 540 nm (methyldopa). Read concentration of noradrenaline or methyldopa from calibration curves prepared in same manner, using concentrations ranging from 30 to 500 µg noradrenaline and from 40 to 600 µg methyldopa, or from 30 to 600 µg and from 40 to 725 µg noradrenaline and methyldopa, respectively, for samples carried through procedure of Ramana and Krishnan Sivamara (10).

Procedure C—Dosage Forms

Determine noradrenaline or methyldopa in tablets, injections, solutions, or syrups by using Procedures A and B after carrying out necessary sample preparations as described in BP and USP methods (7, 8).
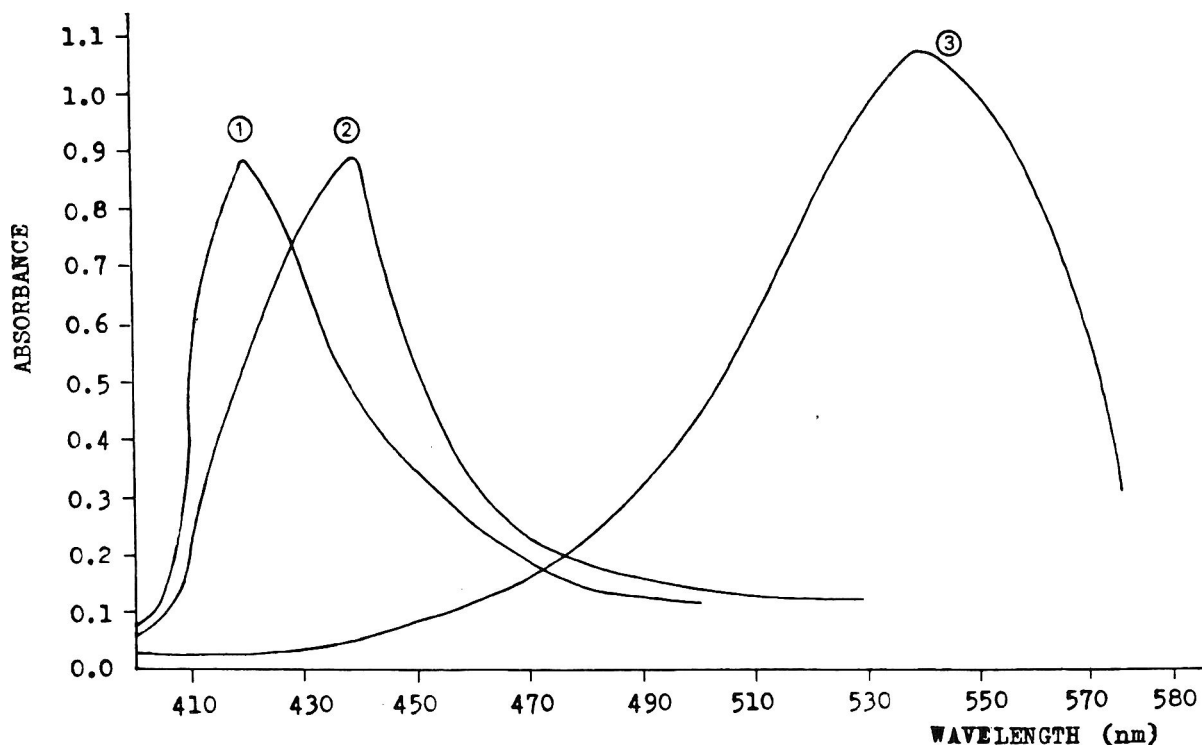
Figure 1.   Absorption spectra for reaction of (1) noradrenaline with p-dimethylaminobenzaldehyde; (2) noradrenaline with p-dimethylaminocinnamal-dehyde; (3) methyldopa with p-dimethylaminobenzaldehyde.
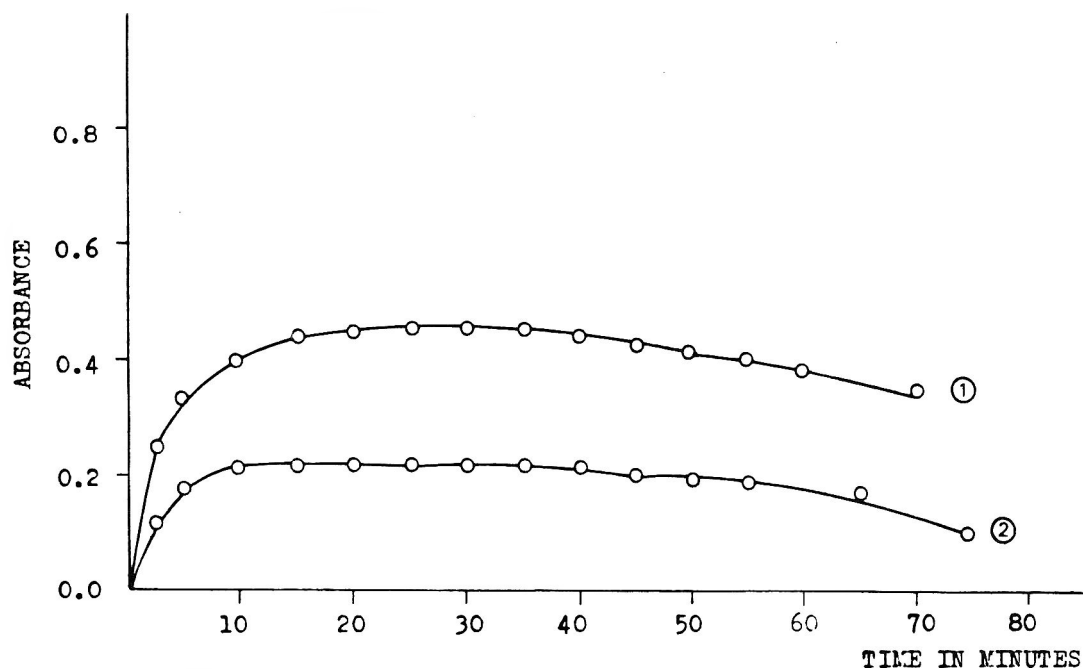
Figure 2.   Effect of time on color produced from reaction between noradrenaline and methyldopa: (1) p-dimethylaminocinnamaldehyde; (2) p-dimethyl-aminobenzaldehyde.

Results and Discussion

To obtain optimum conditions for determination of the sympathomimetic amines, we studied the effect of various factors on the reaction. The results are summarized in the following sections.

Effect of pH

The reaction is enhanced by the use of strong bases, so considerable precautions were taken to prevent the instability of noradrenaline and methyldopa in the alkaline medium. A distinct yellow, orange, or purple color is readily formed with

PDAC or PDAB when the reaction is carried out in the presence (0.5 mL) of 1.0% w/v sodium hydroxide in methanol. The aminochrome derivatives that are produced (9, 11–13) are very unstable; the resulting red color disappears, and brown decomposition products are obtained in 35 min at higher alkali concentrations (14, 15). A more sensitive and deep coloration is obtained in the presence of pH 4.6 acetate buffer or 50% sodium acetate in methanol. Water has been observed to adversely affect the reaction of aminochrome derivatives with PDAC (10, 14). The use of methanol containing water in 50% sodium acetate reagent caused the
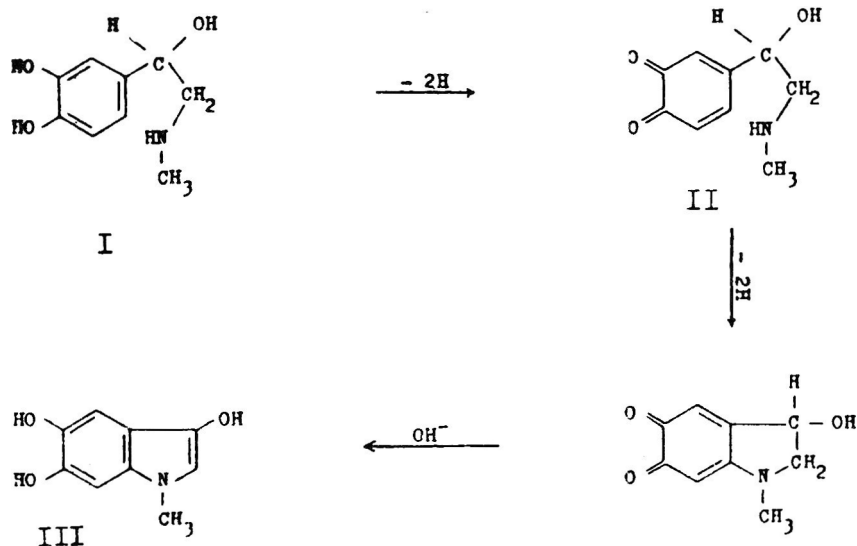
Scheme 1. Reaction of adrenaline (I) under alkaline conditions to produce adrenochrome and the end product leucoadrenochrome (III).
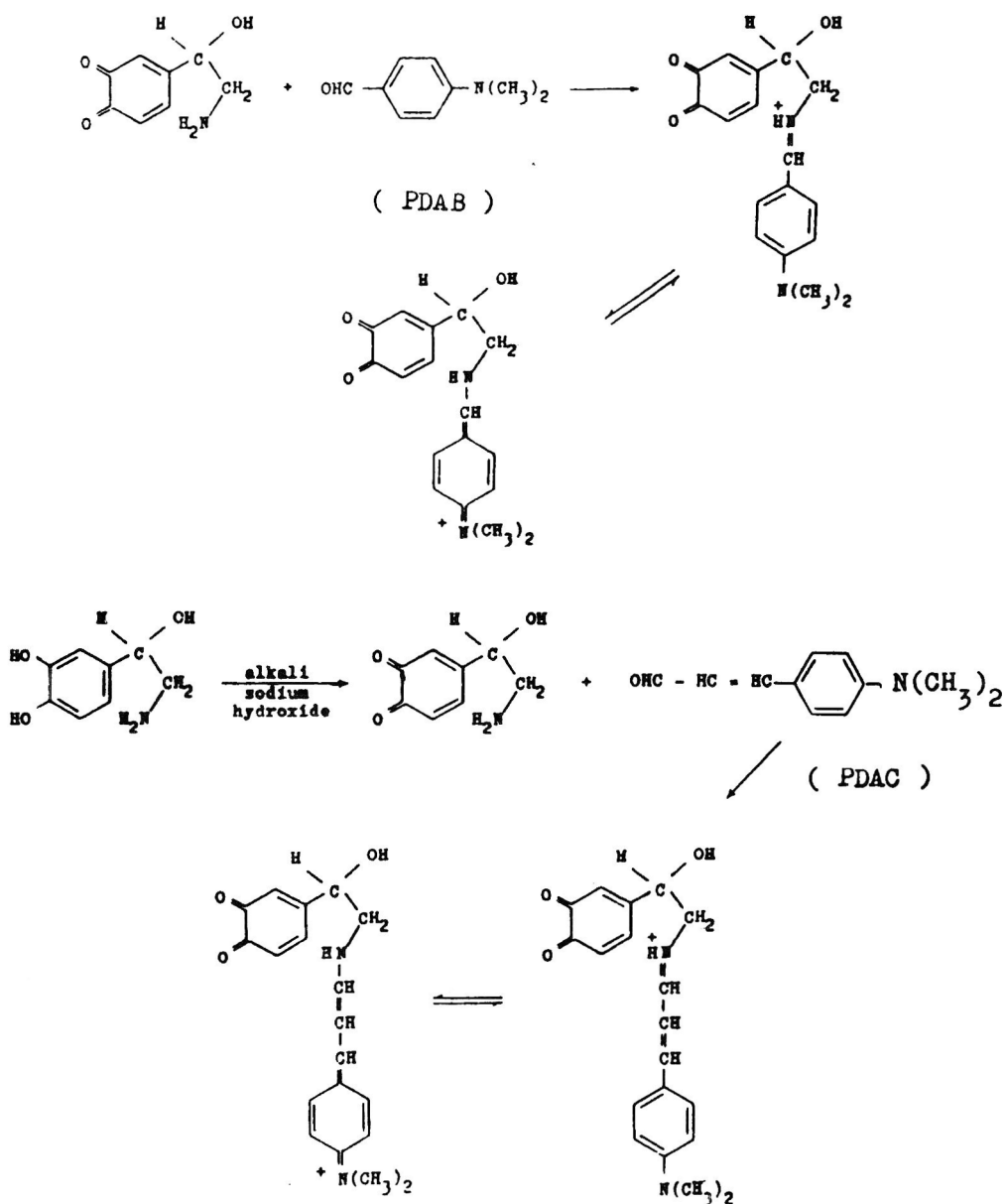
Scheme 2. Reactions of adrenochrome with *p*-dimethylaminobenzaldehyde (top) and *p*-dimethylaminocinnamaldehyde (bottom) to produce adrenochrome *p*-dimethylaminobenzilidene and adrenochrome *p*-dimethylaminocinnamylidene, respectively.

Table 1.   Determination of noradrenaline, noradrenaline in presence of adrenaline, and methyldopa, using p-dimethylaminocinnamaldehyde and p-dimethylaminobenzaldehyde reagents[a,b]

| | | | PDAC reagent | | | PDAB reagent | | | | | |
| | | | | | | Ramana Rao method (10) | | | Proposed method | | |
Noradren., μg taken	Adren., μg added to noradren.	Methyldopa, μg taken	Noradren., % found	Noradren., % found in presence of adren.	Methyldopa, % found	Noradren., % found	Noradren., % found in presence of adren.	Methyldopa, % found	Noradren., % found	Noradren., % found in presence of adren.	Methyldopa, % found
118.3	128.1	84.4	98.36	98.21	96.3	98.762	102.13	98.11	98.82	103.93	98.22
185.9	201.3	168.8	98.24	98.06	98.74	99.47	102.48	98.69	98.67	101.65	98.62
270.4	292.8	295.4	98.97	98.82	99.11	98.55	101.89	99.24	98.87	101.93	99.05
67.6	73.2	379.8	98.32	98.65	99.393	97.29	103.38	99.34	97.95	102.51	99.16
371.8	402.6	464.2	99.0	98.81	99.52	98.76	101.63	99.42	98.67	101.27	99.31
439.4	475.8	590.8	99.29	99.33	99.53	99.22	100.83	99.43	99.22	101.5	99.18
Mean			98.7	98.65	99.1	98.68	102.06	99.04	98.7	102.132	98.93
SD			±0.46	±0.48	±0.507	±0.78	±0.88	±0.55	±0.43	±0.96	±0.428
t-test			0.29	0.27	1.682	1.8	0.07	1.063	0.38	8.88	1.042
F_{ratio}			0.93	0.87	0.12	3.68	0.01	1.6	1.01	3.711	0.46

[a]Each result is the mean of 6 experiments.
[b]Results by official pharmacopoeial method: 98.67 ± 0.073% and 98.731 ± 0.22% for noradrenaline and methyldopa, respectively.
[c]Adrenaline added to noradrenaline to form a mixture in a molar ratio of 1:1.

absorbance values to decrease slowly. The highest sensitivity as well as the maximum stability of the color is observed with sodium acetate in methanol, within 15–45 min, as shown in Figure 2.

Effect of Temperature

The reaction of PDAC with noradrenaline to form Schiff's base is done at an elevated temperature by using a water bath. Gradual destruction of the intense orange color is followed by a distinct decrease in absorbance at 440 nm. Moreover, no reproducible absorption measurements can be obtained, which may be a consequence of competitive side oxidation reactions of the catecholamines at elevated temperatures. In these experiments, however, room temperature is the most favorable condition for carrying out the reaction.

Effect of Reagent Concentrations on Color Intensity

A study was performed to determine the optimum concentration of the PDAC, PDAB, and sodium acetate reagents to develop the maximum color intensity for a given concentration of noradrenaline (183.0 μg). It has been found that 0.5 mL of 0.1% PDAC and 1.0 mL of 1% PDAB are the optimum reagent concentrations and amounts for the assay. Changing the volume of 50% sodium acetate reagent used does not appreciably affect the absorbance readings. In view of the results, 1 mL of 50% sodium acetate in methanol was the concentration of choice for the assay.

Chemistry of the Reaction

Noradrenaline and methyldopa produce color with PDAC and PDAB only after addition of sodium hydroxide. Alkali catalyzes the direct conversion of noradrenaline to noradrenoquinone.

In alkaline medium, adrenaline (I) and isopropylnoradrenaline are rearranged to form the trihydroxyindole derivatives adrenolutine and isopropylnoradrenolutine (14–18). Within the 5 min exposure to alkali sodium hydroxide, the indole ring of noradrenaline does not close but remains in the form of noradrenoquinone (II), in which the nitrogen is active. The compound reacts with the PDAC or PDAB to form the colored conjugate imines that can be measured by spectrophotometry (10, 11, 19, 20). Schemes 1 and 2 show these processes.

Under these experimental conditions, not only adrenaline and isopropylnoradrenaline, but also ephedrine and norephedrine fail to produce any color with these aldehydes. It is

possible, however, that aminochrome derivatives (9, 11–13), which are formed by the effect of alkali on noradrenaline and methyldopa, are responsible for color formation with the aldehydes as illustrated in Schemes 1 and 2.

Precision and Accuracy

Regression analysis of the Beer's plot at 440 nm for the reaction with PDAC reveals excellent correlation ($r = 1$ and 1.033). The calculated r values are greater than the tabulated value, $r_{0.05} = 0.497$ (14 df), so a relationship exists up to a concentration of 60 μg/mL assay solution for both noradrenaline and methyldopa.

Regression analysis for determination with PDAB shows good correlation at both 420 and 540 nm. The calculated value of $r = 1.0$ for both noradrenaline and methyldopa is greater than the tabulated value of $r_{0.05} = 0.532$ (12 df) up to 60 μg/mL for both active ingredients in separate assay solutions.

Table 1 shows the results of analysis of noradrenaline and methyldopa by using PDAC and PDAB reagents. Results compare well with those by official methods (7, 8). Mean percent recoveries and standard deviations are satisfactory. The calculated Student t-test and the F ratio (21) show no significant difference between the results obtained and the official methods.

Table 1 also shows results of analysis of these samples by the procedure of Raman Rao et al. (10). Glycine buffer (pH 1.25) was used and absorbance was measured at 420 nm. Mean percent recoveries and standard deviations agree well with those by official methods.

Conclusions

These procedures offer the advantage that noradrenaline can be determined in the presence of the related compound, adrenaline. Table 1 shows means and standard deviations for determining noradrenaline in the presence of adrenaline in the ratio of 1:1.

Table 2 shows the results for determination of 24 μg noradrenaline in the presence of from 24.0 to 366 μg adrenaline. The results obtained are satisfactory. Student's t-test and F ratio values show no significant difference between the result obtained by the PDAC procedure and the official method. However, the proposed method, using PDAB, shows a significant difference from the official method with respect to the t and F values.

The proposed methods were applied to the analysis of these active ingredients in their dosage forms. The results

Table 2. Determination of noradrenaline in presence of different amounts of adrenaline, using p-dimethylaminocinnamaldehyde and p-dimethylaminobenzaldehyde reagents[a,b]

Noradren., μg taken	Adren., μg added	Noradren., % found	
		PDAC	PDAB
24.0	24.0	98.41	99.05
24.0	109.8	98.63	99.06
24.0	183.0	98.70	99.1
24.0	240.0	98.65	99.12
24.0	292.8	98.67	99.05
24.0	366.0	98.71	99.08
Mean		98.63	99.08
SD		±0.12	±0.012
t-test		1.47	24.84
F_{ratio}		0.59	24.59

[a]Each result is the mean of 6 experiments.
[b]Result by the official pharmacopoeial method: 98.67 ± 0.073% noradrenaline.

Table 3. Recovery (%) of noradrenaline (samples 1, 2, 3) and methyldopa (sample 4) in pharmaceutical preparations, using p-dimethylaminocinnamaldehyde and p-dimethylaminobenzaldehyde reagents[a]

Sample[b]	PDAC reagent	PDAB reagent		
		Ramana Rao method (10)	Proposed method	Official methods (7,8)
1	99.00 ± 0.68	98.88 ± 0.42	98.792 ± 0.371	98.61 ± 0.18
2	98.63 ± 0.591	98.46 ± 0.58	98.55 ± 0.58	98.06 ± 0.07
3	98.07 ± 0.4	98.35 ± 0.32	104.67 ± 0.57	97.99 ± 0.02
4	99.08 ± 0.23	98.94 ± 0.31	99.00 ± 0.45	98.74 ± 0.03

[a]Each result is the mean of 6 experiments with confidence interval (95%).
[b]Label claim of samples: 1, 1 mg noradrenaline/mL (ampule); 2, 0.06 mg noradrenaline/mL (ampule); 3, 0.024 mg each of noradrenaline and adrenaline/mL (ampule); 4, 250 mg methyldopa/tablet.

(Table 3) show good agreement with those obtained by official methods.

REFERENCES

(1) Karnki, O., & Matsuikagaku, I. (1977) *Keisatsukenkysho Hokoku* **30**, 197–200
(2) Benedict, C., & John, B. (1977) *Biochem. Med.* **18**, 455–459
(3) Einosuke, U., et al. (1977) *Clin. Chim. Acta* **80**, 447–453
(4) Stout, R. W. (1976) *Anal. Biochem.* **76**, 330
(5) Yamaguchi, M., Yutani, Y., & Ohkura, K. K. Y. (1979) *Anal. Chim. Acta* **108**, 297–308
(6) Takahashi, S., & Leiv, R. G. (1972) *Clin. Chim. Acta* **36**, 369–372
(7) *British Pharmacopoeia* (1973) University Printing House, Cambridge, UK, pp. 14, 184, 303, 306
(8) *United States Pharmacopeia XX. National Formulary XV* (1980) U.S. Pharmacopeial Convention, Inc., Rockville, MD, pp. 276, 429, 517, 554
(9) Shelton, D., & Henry, S. I. (1974) *J. Pharm. Sci.* **63**, 916
(10) Ramana, G., & Krishnan Sivarama, M. V. (1978) *Indian J. Pharm.* **40**,(1), 18–19
(11) Heller, J., Setlow, R., & Mylon, E. (1951) *Am. J. Physiol.* **166**, 304
(12) Lund, A. (1949) *Acta Pharmacol.* **5**, 231
(13) Green, D., & Richeter, D. (1937) *Biochem. J.* **31**, 596
(14) *Methods of Biochemical Analysis* (1957) Vol. 2, David Glick (Ed.), Interscience Publishers Inc., Div. of John Wiley and Sons, Inc., New York, NY p. 57
(15) Green, S., Mazur, A., & Shorr, E. (1957) *J. Biol. Chem.* **220**, 237–255
(16) Gaddum, J. H. & Schild, H. (1934) *J. Physiol.* **80**, 9
(17) Lund, A. (1949) *Acta Pharmacol.* **5**, 75, 121, 231
(18) Harrison, W. H. (1963) *Arch. Biochem. Biophys.* **101**, 116–130
(19) Wahba Khalil, S. K., & Salama, R. B. (1974) *J. Pharm. Pharmacol.* **26**, 972–974
(20) Yoshitaka, Y., Akiva, S., & Kio, O. (1970) *Bunseki Kagaku* **19**, 1603; *Chem Abstr.* (1971) **74**, 106974d
(21) Hinchen, J. D. (1969) *Practical Statistics for Chemical Research*, Methuen, London, UK, p. 22

Validation of Liquid Chromatographic Method for Assay of Chlorthalidone in Tablet Formulations

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A stability-indicating, reverse phase liquid chromatographic (LC) method was developed for assay of chlorthalidone in tablet formulations. The chromatographic system separates the parent compound from its potential hydrolysis product (4'-chloro-3'-sulfamoyl-2-benzophenone carboxylic acid), which can be quantitated at low levels, and another degradation product (2-chloro-5-(1-methoxy-3-oxo-1-isoindolinyl)benzenesulfonamide), which was found during the experimental work. The procedure can also be used for content uniformity determinations. The general utility of the method was demonstrated by the assay of several product brands. The validated procedure was shown to be accurate, precise, reproducible, and specific.

Chlorthalidone (I), 2-chloro-5-(1-hydroxy-3-oxo-1-isoindolinyl) benzenesulfonamide, is a diuretic-antihypertensive agent. For the determination of I in pharmaceutical dosage forms, a normal phase chromatographic method was reported, which separated the hydrolysis product (II), 4'-chloro-3'-sulfamoyl-2-benzophenone carboxylic acid, on a polyamide column (1). A reverse phase liquid chromatographic (LC) system had been used for the quantitation of I in tablets also containing clonidine hydrochloride; the method was not specific (2). The original USP procedure was a spectrophotometric determination (3); however, it was reported that variable degradation of chlorthalidone occurred during sample preparation (4). The authors used an LC system to separate these degradation products from chlorthalidone; no experimental work was done to show the method sensitivity for the proposed degradation products. The USP procedure for tablets was recently updated to an LC method (5).

A stability-indicating, reverse phase LC method was developed and validated for the assay of I and II in tablet formulations. The method was also used for content uniformity determinations. The general utility of the procedure was demonstrated by the assay of several product brands.

Experimental

Apparatus and Reagents

(a) *Liquid chromatograph.*—Hewlett-Packard 1084B (Hewlett-Packard, Avondale, PA). Chromatographic conditions: injection volume, 25 μL; flow rate, 2.0 mL/min; detector wavelength, 235 nm; detector sensitivity, 0.2 AUFS; column temperature, ambient.

(b) *Column.*—μBondapak C_{18}, 30 cm × 3.9 mm, 10 μm particle size (Waters Associates, Milford, MA) preceded by guard column containing Bondapak C_{18}/Corasil (Waters Associates).

(c) *Mobile phase.*—Methanol–water–acetic acid (35 + 65 + 1).

Sample Preparation

Weigh and finely powder ≥ 20 tablets. Accurately weigh portion of powder, equivalent to ca 25 mg chlorthalidone, and transfer to 50 mL conical centrifuge tube. (For content uniformity determinations, place one tablet directly in centrifuge tube). Add 25.0 mL aliquot of methanol–water (80 +

20). Vigorously shake centrifuge tube 30 min and centrifuge 5 min at 2000 rpm. Pipet 2.0 mL aliquot of supernate into 25 mL volumetric flask and dilute to volume with methanol–water (35 + 65). Filter through 0.5 μm membrane filter before chromatographic analysis.
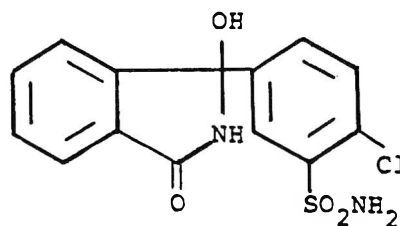
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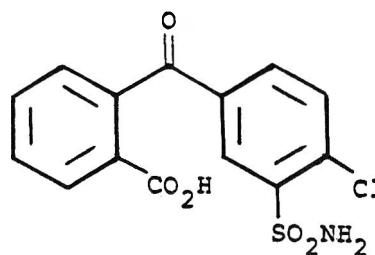
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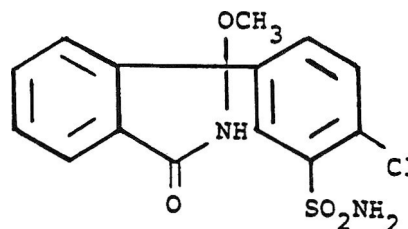
III

Standard Preparation

Accurately weigh ca 25 mg chlorthalidone USP reference standard and transfer to 25 mL volumetric flask. Dissolve and dilute to volume with methanol–water (80 + 20). Transfer 2.0 mL aliquot to 25 mL volumetric flask and dilute to volume with methanol–water (35 + 65.)

Procedure

Inject replicate 25 μL aliquots of standard solution until relative standard deviation for triplicate injections is < 2.0%. When repeatability is established, sample is injected and detected as for reference standard.

Calculate quantity of chlorthalidone in sample by the following formula:

$$\text{mg/tablet} = (R/R') \times C \times (W_a/W) \times F$$

Table 1. LC assay of typical tablet batches of chlorthalidone

Sample	mg Chlorthalidone/tablet		
	Batch 1	Batch 2	Batch 3
1	25.2	24.0	23.7
2	25.1	23.8	23.5
3	25.0	24.1	23.6
4	25.0	24.0	23.3
5	25.0	23.8	23.7
Mean	25.1	23.9	23.6
RSD, %	0.4	0.4	0.8
Label, %	100.4	95.6	94.4

Table 2. LC assay of various product brands of chlorthalidone

Manufacturer	mg Chlorthalidone/tablet	Label, %	II, %[a]
A	24.2	96.6	0.4
A	49.6	99.3	0.3
A	98.1	98.1	0.3
B	24.5	97.8	0.6
B	48.4	96.8	0.4
C	24.3	97.0	0.7
C	49.7	99.4	0.4
D	48.5	97.0	0.4
E[b]	25.1	100.4	0.4

[a]Relative to chlorthalidone content.
[b]Boehringer Ingelheim Ltd, Batch 1.

Figure 1. Representative chromatogram of tablet extract containing I, 2.0 µg (peak A) spiked with 10% each of II (peak B) and III (peak C); see *Experimental* for chromatographic conditions.

where R and R' = peak response (area) for chlorthalidone peak in sample and standard, respectively; C = concentration, mg/mL, of standard preparation; W_a = average tablet weight, mg; W = sample weight, mg; and F = dilution factor for sample.

Results and Discussion

Figure 1 shows a representative chromatogram of tablet extract spiked with 10% II. The acetic acid was added to the mobile phase to optimize the peak shape of II. The chromatographic separation of chlorthalidone and II resulted in k' values of 2.9 and 6.2 and tailing factors of 1.2 and 1.1, respectively.

Detector response (peak area) was linear for I over the range 1.0–3.0 µg injected. A linear regression analysis of the data resulted in a correlation coefficient of 0.9999 with a negative y-intercept value that deviated from the origin by 0.6% (y-intercept/y at the 100% level × 100).

Assays of 5 spiked placebo samples made with I at levels of 59–135% of theory yielded an average recovery of 100.1 ± 0.29%. No placebo interference was observed.

Twenty replicate injections of a tablet sample solution were done over a 10 h period; this resulted in an average peak area with a relative standard deviation of 0.4%. Aside from demonstrating chromatographic precision, the experiment showed the stability of I in the injection solvent over this time period.

In early development work, it was found that chlorthalidone when dissolved in the mobile phase and allowed to stand for approximately 24 h formed a degradation product (III), 2-chloro-5-(1-methoxy-3-oxo-1-isoindolinyl)benzenesulfonamide, which eluted with a k' of 8.4 (Figure 1). This was confirmed by comparison of its retention time and UV spectrum with an authentic sample. Ether formation is known to occur readily when I is reacted with lower chain alcohols in the presence of traces of strong acids (6, 7). Ether formation was avoided by the subsequent deletion of acetic acid from the sample preparation and injection solvents.

To show the overall reproducibility of the method, 5 replicate assays were done on each of 3 typical batches (Table 1). Content uniformity determinations on 10 individual tablets of batch 1 yielded 25.1 ± 0.7 mg I/tablet, which was in agreement with the composite assay. In addition, 5 replicate assays were done on batch 1 using the USP procedure (3); this resulted in 25.2 ± 0.5 mg I/tablet, which was in conformance with the LC data. To show the applicability of the method to various product brands, several tablet formulations were assayed in duplicate; Table 2 summarizes the data.

A system suitability test was routinely used to evaluate the chromatographic system. This involved chromatography of a solution containing I (0.08 mg/mL) and II (0.16 mg/mL), which resulted in peaks of approximately equal response. The resolution between the 2 peaks was greater than 6.4.

Water was included in the extraction solvent, methanol-water (80 + 20), to aid disintegration of the tablets, especially for content uniformity determinations. The higher ratio of methanol in the extraction solvent, relative to the mobile phase composition, was necessary to ensure extraction efficiency of I from the tablet matrix. The sample was further diluted with the appropriate ratio of methanol-water to adjust the sample solvent to the mobile phase composition.

The feasibility of the quantitation of II was investigated. To detect levels of 0.1% relative to the parent compound, the

tablet extract from the first dilution of the sample preparation (containing 1.0 mg I/mL) was chromatographed at the appropriate sensitivity. Validation work affirmed the applicability of the method for determination of this compound. Peak area was linear for II over the range 0.05–1.2 μg (0.2–5.0%). A linear regression analysis of the data resulted in a 0.9998 correlation coefficient with a positive y-intercept value that deviated from the origin by 1.5% (y-intercept/y at the 2% level × 100). Using the method of standard addition, 5 tablet samples containing 0.4% II were reassayed after addition of 1–5% II, with an average recovery of 99.9 ± 2.6%. Twenty replicate injections of a tablet extract containing 0.4% II, done over a 10 h period, resulted in an average peak area with a relative standard deviation of 2.8%. The product brands were assayed in duplicate for II, and the results are shown in Table 2.

Analytical results show that this LC method is accurate, precise, reproducible, and specific. The general applicability of the procedure to different formulations was evidenced by the data obtained on various products.

REFERENCES

(1) O'Hare, M. J., Tan, E.. & Moody, J. (1979) J. Pharm. Sci. 68, 106–108
(2) Walters, S. M., & Stonys, D. B. (1983) J. Chromatogr. Sci. 21, 43–45
(3) U.S. Pharmacopeia (1980) 20th Rev., U.S. Pharmacopeial Convention, Rockville, MD, p. 147
(4) Bauer, J., Quick, J., Krogh, S., & Shada, D. (1983) J. Pharm. Sci. 72, 924–928
(5) U.S. Pharmacopeia (1980) 20th Rev., Addendum to Supplement 4, U.S. Pharmacopeial Convention, Rockville, MD, p. 818
(6) Graf, W., Girod, E., Schmid, E., & Stoll, W. G. (1959) Helv. Chim. Acta 42, 1085
(7) Auterhoff, H., & Wessinger, W. (1979) Arch. Pharm. 312, 876

Colorimetric Determination of Stanozolol in Pharmaceutical Formulations

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In acidic medium, stanozolol reacts with phenoldisulfonic acid to form a stable yellow chromophore, which is quantitated by spectrophotometry at 385 nm. The reaction gives a linear response at concentrations from 5 to 50 μg/mL. The method is suitable for routine analytical control of stanozolol and its formulations.

Stanozolol, 17-methyl-2'H-androst-2-eno(3,2-c)-pyrazol-17-ol, is used for anti-anemia therapy and as an anabolic steroid. The official compendia (1, 2) describe nonaqueous titrimetry for quantitation of the pure drug and colorimetric or UV spectrophotometric methods for tablets. A survey of the literature revealed relatively few methods for estimation of stanozolol. Magin (3) described a gas chromatographic method for determination of stanozolol and its major metabolites. Lanto et al. (4) described a highly sensitive method for estimation of stanozolol in biological fluids, based on isotope dilution-mass fragmentography. Other reported methods involve combined gas chromatography–mass spectrometry (5–7), radioimmunoassay (8–10), and mass fragmentography (11).

The colorimetric method (1) stipulates a time-consuming procedure that involves heating for 45 min in a water bath. Excipients in injection and suspension formulations interfere in the UV spectrophotometric method (2).

This paper describes a colorimetric determination based on a simple, sensitive reaction of stanozolol with phenoldisulfonic acid in acidic medium. This is a typical Kober reaction (12) in which the golden yellow chromophere that is formed exhibits maximum extinction at 385 mm. The reaction can be used for analytical control of stanozolol and its marketed formulations, without interference from excipients (thiomersal, Tween 80, sodium chloride, butyl-p-hydroxy benzoate, methyl and propyl parabens, sodium saccharin, sorbitol, propylene glycol, magnesium stearate, and the colors amaranth and erythrosine).

METHOD

Apparatus and Reagents

(a) Spectrophotometer.—Beckman Model DU.
(b) Chemicals.—All AnalaR grade reagents. Glacial acetic acid; phenoldisulfonic acid (BDH, England); methanol; chloroform.
(c) Stanozolol standard solution.—Dissolve suitable quantity of stanozolol in glacial acetic acid and dilute quantitatively and stepwise with glacial acetic acid to concentration of 250 μg/mL.

Calibration Curve

Transfer 0.5, 1.0, 1.5, 2.0, up to 5.0 mL stanozolol standard solution into separate 25 mL volumetric flasks, and add sufficient glacial acetic acid to make total volume of 5.0 mL. Include flask containing glacial acetic acid as blank. To each flask add 2.0 mL phenoldisulfonic acid reagent. Mix and let flasks stand 15 min at room temperature (24–28°C). Dilute solution to volume with glacial acetic acid, mix, and determine absorbance of each solution in 1 cm cell at 385 nm against the blank. Figure 1 shows absorption spectrum of stanozolol chromophore. Plot absorbances at 385 nm vs concentrations of stanozolol.

Sample Preparation and Assay

(a) Tablets.—Weigh 20 stanozolol tablets to determine average tablet weight, and then finely powder tablets. Transfer an accurately weighed portion of powder, equivalent to ca 25 mg stanozolol, into 200 mL beaker. Add ca 50 mL glacial acetic acid, and heat 15 min on steam bath with frequent swirling. Filter into 100 mL volumetric flask. Complete extraction with 2 additional 20 mL portions of glacial acetic acid, add filtrates to same volumetric flask, and dilute contents to volume with glacial acetic acid. Mix thoroughly, pipet 2.5 mL solution into 25 mL volumetric flask, and proceed as for Calibration Curve.

(b) Injections.—For assay, accurately measure volume of injection equivalent to 25 mg stanozolol. Dilute sample por-

Table 1.   Estimation of stanozolol in pharmaceutical formulations by official and proposed methods

Sample[a]	Label claim, mg	Found, mg		Std dev.	Coeff. of var., %	Rec., %
		Official method	Proposed method[b]			
T	2/tab	1.998	2.004	0.0054	0.27	100.58
I	50/mL	—	50.058	0.1106	0.22	100.34
S	0.4/mL	—	0.401	0.0043	1.07	99.27

[a]T = tablet, I = injection; S = suspension.
[b]Average of 6 determinations.
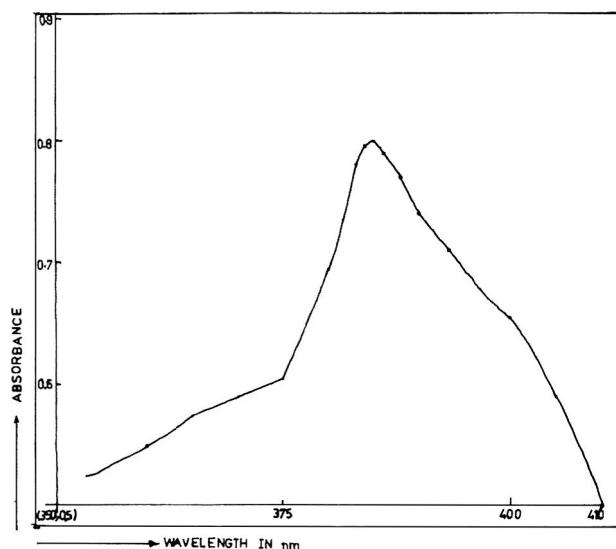
Figure 1.   Absorption spectrum of stanozolol chromophore developed by reaction.

tion quantitatively and stepwise with glacial acetic acid to 250 µg/mL. Pipet 2.5 mL of solution into 25 mL volumetric flask, and proceed as for Calibration Curve.

(c) Suspension.—Pipet 10 mL of well mixed suspension into 125 mL glass-stopper, conical flask that contains 60 mL methanol–chloroform (1 + 1). Let pipet drain thoroughly, and rinse with two 5 mL portions methanol–chloroform (1 + 1). Reflux mixture under condenser for 30 min; cool, and decant supernate into 100 mL volumetric flask. Rinse flask and solid residue with two 5 mL portions of methanol–chloroform (1 + 1), transfer rinse to volumetric flask and dilute to volume with same solvent. Pipet 20 mL of dilution into 250 mL glass-stopper graduated cylinder, and add 100 mL water and 40 mL chloroform. Shake 2 min, then let solution separate (total chloroform volume will be 50 mL). Aspirate upper aqueous layer and centrifuge lower chloroform layer. Transfer 30 mL of solution (chloroform layer) into beaker and cautiously evaporate chloroform. Dissolve residue in 5 mL glacial acetic acid and use solution for assay as for Calibration Curve.

Recovery Study

The reliability and suitability of the proposed method were confirmed by adding known quantities of stanozolol to various preanalyzed stanozolol formulations, and analyzing the mixtures by the proposed method.

The percent recovery of stanozolol (Table 1) was calculated by using the formula

$$\% \text{ recovery} = \frac{N(\Sigma XY) - (\Sigma X)(\Sigma Y)}{(\Sigma X^2) - (\Sigma X)^2} \times 100$$

where X = mg stanozolol added; Y = mg stanozolol found; and N = total number of observations.

Validation Study

This study was intended to provide an estimate of assay accuracy and precision and was not concerned with assay sensitivity, response linearity, or with the cause(s) of observed deviations for assay application. The validation samples, apart from the drug to be assayed, were composed of the other formula components in the prescribed formula composition. Three pairs of samples for duplicate determinations were prepared by qualified personnel on a coded "blind" basis as follows:

(A) Two samples contained the nominal product formula quantity of stanozolol.

(B) Two samples contained an amount equal to the nominal product formula minus 1.5 times the difference between the nominal formula quantity and the upper assay specification limit.

(C) Two samples contained an amount equal to the nominal formula plus 1.5 times the difference between the nominal formula quantity and the upper assay specification limit.

Each analyst carried out 2 determinations on each pair of the 3 drug levels by the proposed method. The results of analyses were reported as percent of formula quantity. Calculations for drug level (A) prepared as shown above were as follows:

$$\text{Av. \% found } (Z_A) = \frac{a_1 + a_2 + a_3 \ldots \ldots \ldots + a_5}{5}$$

% Relative deviation (D_A)

$$= \frac{|a_1 - Z_A| + |a_2 - Z_A| + \ldots \ldots + |a_5 - Z_A|}{5} \times \frac{100}{Z_A}$$

where | | indicates taking absolute values (ignore the sign of resultant subtraction).

% Relative error (E_A) (accuracy)

$$= \frac{(a_1 - X_A) + (a_2 - X_A) + \ldots \ldots + (a_5 - X_A)}{5} \times \frac{100}{X_A}$$

where $a_1, a_2, \ldots a_5$ are each % of formula quantity of stanozolol found by Analyst No. 1, 2, . . . 5, respectively, and X_A indicates % formula quantity of stanozolol added. Calculations for the other drug levels B and C were similarly computed.

The validation study on the proposed method gave promising results (Table 2).

Results and Discussion

The absorption (385 nm) properties of the colored species as well as the influence of different parameters on color development were studied to determine the optimal conditions for the assay procedure. The course of the reaction was studied as a function of concentration and volume of reagent, initial reaction time, and stability of reaction.

Effect of concentration and volume of reagent.—The reaction requires 2 mL phenoldisulfonic acid (25% w/v) for max-

Table 2. Validation study of proposed method for estimation of stanozolol in formulations[a]

| Drug level | Added (X) | Found (a), by analyst[b] | | | | | Av. (Z) | % Rel. dev. (precision) (D) | % Rel. error (accuracy) (E) |
		1	2	3	4	5			
A_T	85	84.88	85.50	85.20	84.90	85.72	85.24	0.27	+0.28
B_T	100	99.80	99.90	100.90	100.08	98.57	99.85	0.53	−0.75
C_T	115	115.30	114.95	115.05	114.99	115.71	115.20	0.21	+0.17
A_I	85	84.95	85.10	85.00	85.60	86.35	85.40	0.54	+0.48
B_I	100	99.10	99.95	101.05	100.00	99.50	99.92	0.50	−0.08
C_I	115	114.90	115.05	114.85	115.12	114.85	114.95	0.09	−0.04
A_S	85	84.80	85.20	86.10	84.90	86.70	85.54	0.81	−0.64
B_S	100	99.20	99.80	100.05	99.75	100.10	99.78	0.24	−0.22
C_S	115	114.30	115.10	114.85	114.79	115.05	114.82	0.19	−0.16

[a] T = tablet; I = injection; S = suspension. See text for description of A, B, C sample preparation and for definitions of terms.
[b] Two determinations on each sample.
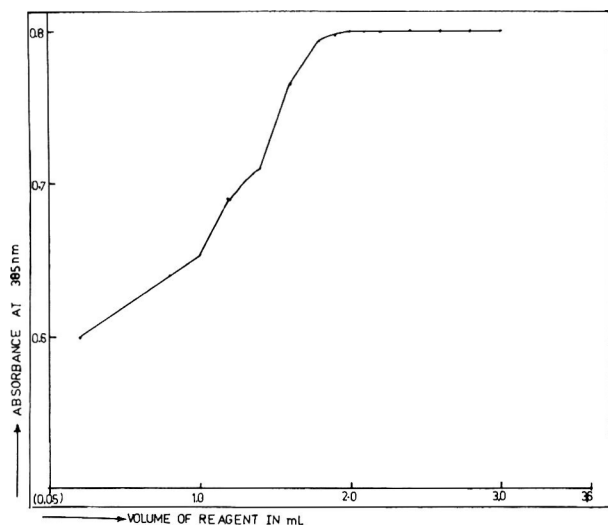
Figure 2. Volume of 25% w/v phenoldisulfonic acid in sulfuric acid required to develop maximum color at 385 nm.
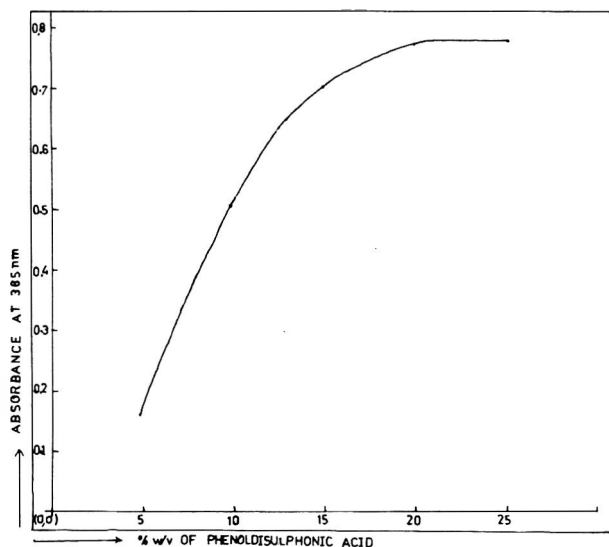
Figure 4. Effect on absorbance (385 nm) of concentration of reagent in sulfuric acid.
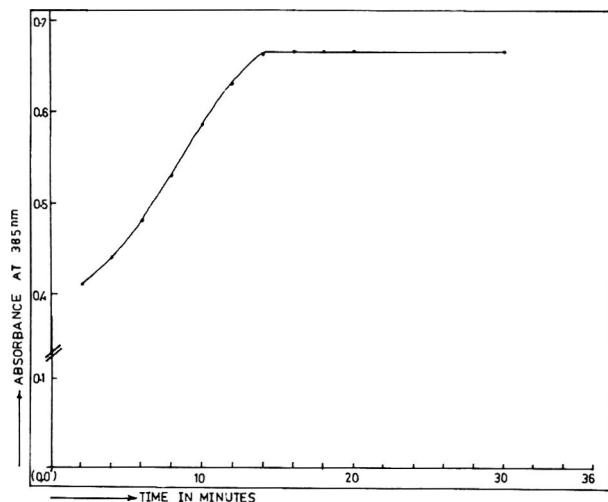
Figure 3. Initial time (at 24–28°C) required for completion of reaction at 385 nm.

Figure 5. Effect of time on absorbance of reaction components at 385 nm.

imum color development. Further increase in volume of the reagent did not reveal any apparent change in color intensity (Figure 2).

Effect of initial reaction time.—Optimum results are obtained when 2 mL phenoldisulfonic acid (25% w/v) is allowed to react with stanozolol for the initial time of 15 min (Figure 3).

Effect of varying reagent concentration.—Absorbance of the chromophore increases as the concentration of phenoldisulfonic acid increases. This effect levels off at a concen-

tration of about 25% w/v. which is specified in the assay. A larger volume of reagent must be used if the reagent is diluted with sulfuric acid (Figure 4).

Stability of reaction.—The golden yellow chromophore formed was stable for 3 h at room temperature (24–28°C), and then exhibited a gradual decrease in absorbance (Figure 5).

Beer's law.—Under the proposed experimental conditions, the relationship between absorbance and concentration of stanozolol is linear between 5 and 50 μg/mL.
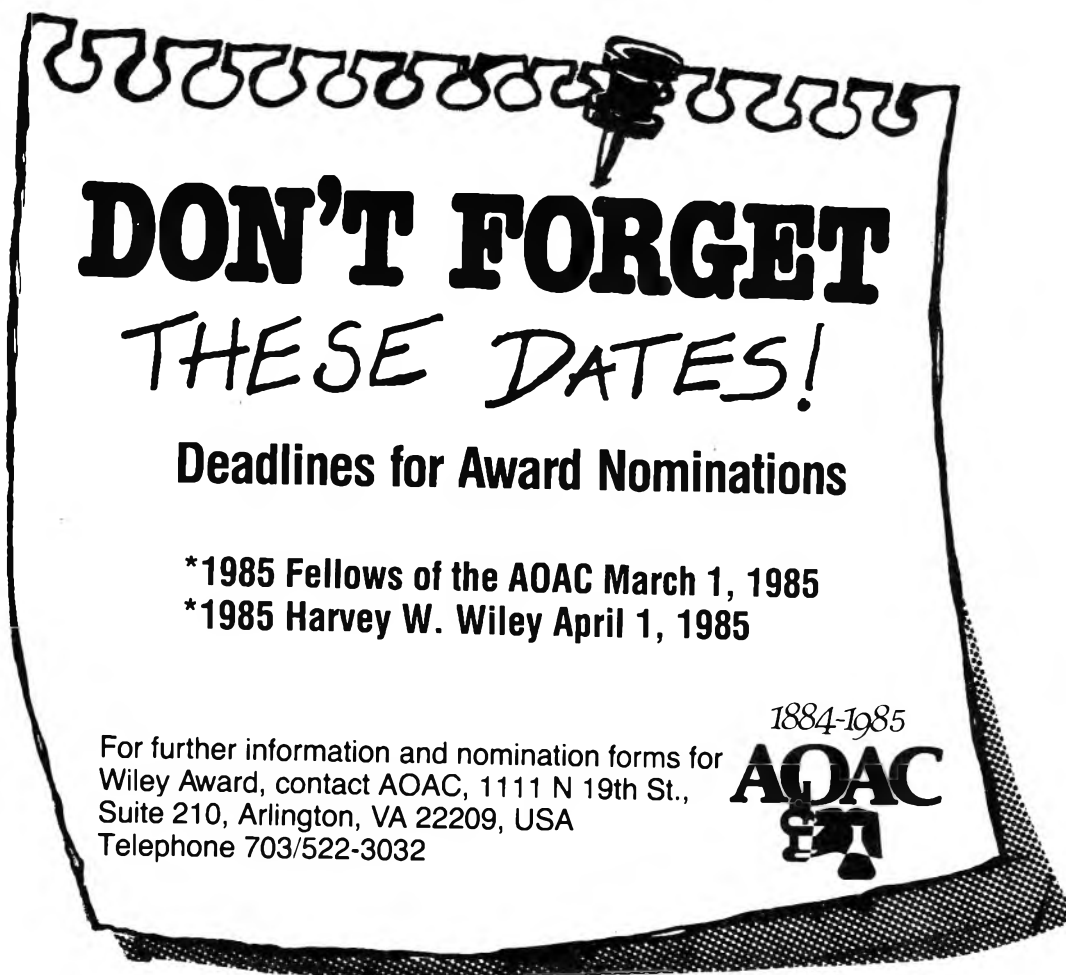
This colorimetric method provides a useful assay procedure for routine quality control of stanozolol in pharmaceutical formulations.

Acknowledgment

REFERENCES

(1) *National Formulary XIV* (1975) U.S. Pharmaceutical Convention. Rockville, MD, pp. 657–658
(2) *U.S. Pharmacopeia XX* (1980) U.S. Pharmacopeial Convention, Rockville, MD, pp. 741–742
(3) Magin, D. F. (1975) *J. Chromatogr.* **115**, 687–689
(4) Lanto, O., Bjoerkhem, I., Ek, H., & Johnston, D. (1981) *J. Steroid Biochem.* **14**(8), 721–727; *Chem. Abstr.* (1981) **95**, 199902k
(5) Brooks, C. J. W., et al. (1970) *Adv. Chromatogr.* **6**, 262–272; *Chem. Abstr.* (1971) **74**, 94842d
(6) Ward. R. J., Lawson, A. M., & Shackleton, C. H. L. (1976) *Mass Spectrom. Drug Metab.* 465–474; *Chem. Abstr.* (1978) **88**, 34061c
(7) Bertrand, M., Massem, R., & Dugal. R. (1978) *Farm. Tijdschr. Belf.* **55**(3), 85–101; *Chem. Abstr.* (1979) **90**, 115928j
(8) Ward, R. J., Shackleton, C. H. L., & Lawson, A. M. (1975) *Br. J. Sports Med.* **9**(2), 93–97; *Chem. Abstr.* (1978) **89**, 159645y
(9) Dugal, R., Dupuis, C., & Bertrand, M. (1977) *Br. J. Sports Med.* **11**(4), 162–169; *Chem. Abstr.* (1978) **89**, 142818j
(10) Hampl, R., Picha, J., Chendela, B., & Starka, L. (1978) *J. Clin. Chem. Clin. Biochem.* **16**(5), 279–282; *Chem. Abstr.* (1978) **89**, 103108h
(11) Rumboldt, Z., & Barbir, Z. (1980) *Lijec. Vjesn.* **102**(5), 261–268; *Chem. Abstr.* (1981) **94**, 115313n
(12) Kober, S. (1938) *Biochem. J.* **32**, 357

DAIRY PRODUCTS

Determination of Phosphorus in Processed Cheese: Collaborative Study

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A second interlaboratory collaborative study of the determination of
phosphorus in processed cheese products by the molybdenum blue
method verifies that this method is prone to producing a laboratory-
induced systematic error. It would be useless to continue to make minor
modifications in the details of the method, which will improve only the
within-laboratory precision, until an accuracy control of the final mea-
surement step is incorporated into the method.

A collaborative study of the determination of phosphorus in
processed cheese by the molybdenum blue colorimetric method
(1) of the International Organization for Standardization (ISO)
was conducted by Brzenk and Krett (2). That study con-
cluded that the method required further investigation to reduce
the between-laboratory error component. The International
Dairy Federation (IDF) modified the method by changing the
reducing agent from hydrazine to ascorbic acid and by shifting
the wavelength for reading the developed color from 700 to
820 nm. A further collaborative study was then conducted to
determine the effect of these modifications. This paper reports
the results of the second study.

Experimental

Products

Twelve processed cheese products, prepared from various
varieties of cheese, were sent to 10 laboratories to be ana-
lyzed in duplicate by each of 2 methods. The original cheeses,
of unknown phosphorus content, contained 15–55% fat in the
solids and 40–57% solids. Nine of the products contained
2.7–3.0% added disodium phosphate, and products 2, 9, and
10 contained 1.8–2.9% added polyphosphate.

Method I. Revised ISO Method

This method consisted of digestion of the cheese with sul-
furic acid and hydrogen peroxide, formation of molybdenum
blue by addition of sodium molybdate and ascorbic acid, and
measurement of the blue color at 820 nm.

Method II. ISO 2962, Reference Method

For determination of phosphorous content in cheese and
processed cheese products, also reproduced as IDF Standard
33A:1971 (3). This method is the same as Method I except
that the reduction is performed with hydrazine, and the color
is measured at 700 nm.

Statistical Analysis

The original data were entered into the Food and Drug
Administration computer system and analyzed by the statis-
tical program FDACHEMIST (4), that is written in APL

language and calculates the mean, median, standard devia-
tions, coefficients of variation (CV) (within- and among-lab-
oratories), and ISO repeatability (r) and reproducibility (R)
intervals; flags outliers by the Dixon (1- and 2-tail), Grubbs,
and Cochran tests; and produces a graph of the data input.
Auxiliary programs are available for the performance of
analysis of variance, rank sum test, and removal of outlying
values and laboratories.

Results

Because a complete 2-way analysis of variance of the data
showed that there was no significant difference between
methods, the duplicate results from each laboratory by each
method were combined to give 4 values from each laboratory
at each level. Because the within-laboratory variability is
considerably smaller than the reproducibility variability (ratio
of within- to between-variabilities of 0.38 with no outliers
removed), the original individual values are not given, but
are available on request from the authors. The averages of
the 4 values for each laboratory at each level are plotted in
Figure 1 as percent phosphorus (absolute) on the Y-axis and
the cheese number (product) on the X-axis. At the bottom of
the figure on the same absolute phosphorus scale is given the
ISO reproducibility interval, R (5), read on the %P (absolute)
scale on the left, and the AOAC CV_x (6), read on the $%CV_x$
(relative) scale on the right, material by material. The ISO
intervals are prediction intervals and are obtained from the
within- and between-laboratories standard deviation by mul-
tiplying the corresponding s_o and s_x by the factor $2\sqrt{2}$. The
upper part of the figure includes all data for the product
averages with no outliers removed and the lower part contains
R and CV_x with and without Laboratories 11 (highest) and 3
(lowest) removed as rank sum outliers.

Table 1 provides a summary of the results, material by
material, with and without the 2 rank sum outlying labora-
tories removed. The first column gives the material number,
the second column gives the average percent phosphorus,
and the third and fourth columns show the number of deter-
minations and laboratories supplying the data for that row,
respectively. In this case, dividing the number of determi-
nations by the number of laboratories gives 4 replicates per
laboratory. Columns 5 and 6 indicate that the data were
calculated with the indicated percent and kind of outliers
removed, 0/0 indicating no outliers removed. The second line
for each material shows that, with the 2 outlying laboratories
removed as rank sum (RS) outliers, 20% of the data has been
omitted. The final 2 columns under coefficients of variation
give the repeatability and reproducibility CVs; the one on the
left is the CV with outliers retained and the one on the right,
with outliers removed. All of the data are averaged or summed
at the bottom of the table, with the average CVs accompanied
by the standard deviation of the 12 CVs averaged for each
column merely to indicate the dispersion of results around
the mean CV for that column.

Table 1. Design and precison parameters for determination of phosphorus in processed cheese products by molybdenum blue method (see text for explanation of column and row entries)

Material	Av. % P	Detns	Labs	Outliers %	Outliers Kind	CV_o		CV_x	
1	0.932	40	10	0	0	1.68		6.89	
	0.939	32	8	20	RS		1.65		2.92
2	0.861	40	10	0	0	4.26		6.99	
	0.863	32	8	20	RS		1.84		3.29
3	0.927	40	10	0	0	1.82		6.29	
	0.929	32	8	20	RS		1.46		3.24
4	0.877	40	10	0	0	1.92		5.71	
	0.874	32	8	20	RS		1.77		2.90
5	0.892	40	10	0	0	1.70		6.33	
	0.890	32	8	20	RS		1.78		2.54
6	0.981	40	10	0	0	2.22		5.98	
	0.987	32	8	20	RS		1.57		3.11
7	0.974	40	10	0	0	3.02		9.48	
	0.987	32	8	20	RS		2.04		3.88
8	0.813	40	10	0	0	2.86		7.15	
	0.810	32	8	20	RS		2.15		3.29
9	0.840	40	10	0	0	2.05		7.16	
	0.840	32	8	20	RS		1.92		4.32
10	0.851	40	10	0	0	2.23		5.49	
	0.853	32	8	20	RS		1.42		3.51
11	1.030	40	10	0	0	3.44		6.83	
	1.031	32	8	20	RS		3.23		4.47
12	1.034	40	10	0	0	3.42		6.60	
	1.027	32	8	20	RS		1.31		3.54
Av. or	0.919	(384)	8	20	RS		1.85 ± 0.50		3.42 ± 0.57
(total)	0.919	(480)	10	0	0	2.55 ± 0.83		6.74 ± 1.02	

Discussion

Figure 1 clearly shows that each laboratory maintains a more or less consistent systematic error in the examination of all the cheese products. This is most clearly evident with Laboratories 3 and 11, which provide the lowest and highest values at all levels, respectively. These laboratories are obvious rank sum outliers at a confidence level greater than 99.9%; therefore, we are clearly justified in removing these 2 laboratories as consistent rank sum outliers and recalculating the parameters for the remaining laboratories whose values would then be relatively close together. The reduction of the CV_x (or R) by 50% and the appearance of the data on the graph classify these outliers as "influential." However, each of the remaining 8 laboratories also contains a smaller yet consistent systematic error in relationship to each other (or to the mean of each material). For example, Laboratory 7 is almost always the second highest laboratory and Laboratory 8 is almost always the second lowest laboratory.

This is almost exactly the same pattern which was exhibited in the previous study in Brzenk and Krett (2) with 7 laboratories at 5 levels. In that study, 2 of the 7 laboratories were also high and low rank sum outliers, respectively, but at a significance level closer to 95%. The CV_o and CV_x of the previous study were 2.4 and 9.1%, respectively (the CV_x of 7.6% in the original paper has been recalculated, because apparently the laboratory-sample interaction term was omitted from the final calculation); the corresponding r and R are 0.043 and 0.170% (absolute), respectively.

If the 2 rank sum outliers are removed, the method has acceptable precision parameters of about 0.05% (absolute) for repeatability and 0.09% (absolute) for reproducibility, or in terms of CVs, about 1.8 and 3.4% (relative), respectively. These are acceptable for a 1% concentration level (7). Nevertheless, the pattern exhibited by the individual laboratories reveals the presence of an uncontrolled systematic error on the part of each laboratory. Neither Laboratory 3 nor Laboratory 11 was aware of their position in relationship to the other laboratories until the results of the study were transmitted to them. Without some reference point, no laboratory is able to determine whether or not their values are correct, or if they are exhibiting an invisible (to them) systematic error.

There is an alternative interpretation of the systematic data patterns for laboratories. If the use of the rank sum test is not considered acceptable in this situation, but rather the extreme values are merely an extreme manifestation of the random systematic error pattern between laboratories, and the extreme values are part of the normal population, then the precision parameters are too large for enforcement purposes. Something must be done to reduce the type of error exhibited in these 2 studies.

It is essential, therefore, that some accuracy control be incorporated into this method. The simplest such control would be to specify the use of a reference phosphorus material as a standard such as the National Bureau of Standards (NBS) SRM 186-I potassium dihydrogen phosphate. The method should incorporate an absolute or consensus absorptivity value for a phosphate standard solution of specified concentration with a statement that if this absorptivity plus or minus a reasonable tolerance is not attained, the spectrophotometer should be checked against an NBS certified reference glass of known absorbance and adjusted accordingly before further measurements are made. The wavelength scale should be simultaneously checked with a reference lamp. The absorptivity of molybdenum blue at 830 nm is given as 2.68 $\times$ 10⁵ for 1 mol/L in a 1 cm cell (8). An alternative statement could be in terms of the absorbance difference or the slope of the calibration curve for a given concentration range, with a reasonable tolerance. The possibility of error in the preparation of the standard solution should not be overlooked.

The differences in fat or solids content or the nature of the phosphate added had no significant effect on the estimated phosphorus content. The fact that the reproducibility interval, R, and the CV_x parallel each other is not unusual because

P in Cheese
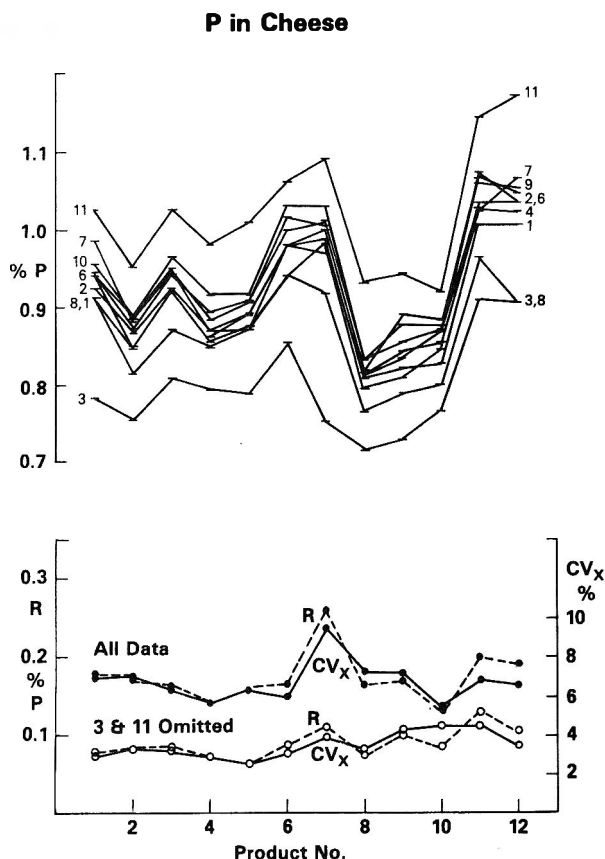
Figure 1.   Upper curves: Average phosphorus content (%) of processed cheese products calculated from 4 values from each of 10 laboratories for each of 12 products. Laboratory 5 was not assigned. Lower curves: ISO reproducibility interval (R) is read on the left scale and AOAC reproducibility coefficient of variation (CVₓ) is read on right scale, with and without rank sum laboratory systematic outliers (Laboratories 3 and 11) removed.

all of the products contain about the same phosphorus content of 0.9%.

Dixon and Cochran outliers, often at the 99% confidence level, are flagged frequently, primarily from Laboratory 3. The Dixon flags should be ignored, however, in the presence of this type of systematic error, because it would be incongruous to determine whether a value from Laboratory 3 or 11 is an outlier depending on the relative position of the next to the last opposite point in the set (usually from Laboratory 7 or 8), as is required in this outlier test. Even if the rank sum flag were to be ignored, elimination of the laboratories on the basis of the Dixon test (usually initially from Laboratory 3) or the Cochran test (also usually from Laboratory 3) would result in the corresponding value from Laboratory 11 becom-

ing a flagged outlier, ultimately achieving, in many cases, the same end result as the rank sum test. Because of the consistent presence of rank sum outliers, the sporadic appearance of Dixon and Cochran outliers would be of only secondary importance, and, therefore, these flags are ignored in this interpretation.

Conclusion and Recommendations

A second collaborative study of the molybdenum blue method for the determination of phosphorus in cheese and cheese products reinforced the original conclusion of the presence in the method of a systematic laboratory-related error. It is recommended that a new study be initiated, in which all laboratories would measure the absorptivity of a standard phosphate solution, using spectrophotometers with scales that have been checked against designated standards to incorporate an accuracy control into the method. Then the study of the determination of phosphorus in cheese should be repeated, incorporating some blind duplicates into the design.

Acknowledgments

REFERENCES

(1) ISO 2962-1974. Cheese and processed cheese products—Determination of phosphorus content (Reference method); available from International Standardization Organization, CH-1211 Geneva 20, Switzerland, and national standardization organizations
(2) Brzenk H. R., & Krett, O. J. (1976) *J. Assoc. Off. Anal. Chem.* **59**, 1142–1145
(3) IDF Standard 33A:1971. Cheese and processed cheese products—Determination of total phosphorus content (Photometric method); available from International Dairy Federation, Square Vergote 41, B-1040 Brussels, Belgium
(4) Horwitz, W., & Albert, R. H. (1984) *J. Assoc. Off. Anal. Chem.* **67**, 81–90
(5) ISO 5725-1981. Precision of test methods—Determination of repeatability and reproducibility by interlaboratory tests; available from International Organization of Standardization, Geneva, Switzerland, and from national standardization organizations
(6) Youden, W. J., & Steiner, E. H. (1975) *Statistical Manual of the AOAC*, AOAC, Arlington, VA
(7) Horwitz, W. (1982) *J. Assoc. Off. Anal. Chem.* **65**, 525–530
(8) Meites L. (1963) *Handbook of Analytical Chemistry*, McGraw-Hill Book Co., New York, NY, pp. 6–37

FORENSIC SCIENCES

Paper Chromatographic Method for Identification and Semiquantitative Determination of Pyridine and α-Picoline in Denatured Spirit, French Polish, and their Derived Preparations

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A paper chromatographic method is described for determination of pyridine and α-picoline in ordinary denatured spirit, french polish, and their derived illicit liquors. The method is based on conversion of the pyridine bases to their salts (sulfate or hydrochloride). The salt residue is subjected to 2-dimensional paper chromatography on filter paper, using methanol–butanol–acetic acid–ethyl acetate (20 + 10 + 1 + 10) as developing solvents, and is sprayed uniformly with sodium rhodizonate followed by barium chloride (for sulfate salts), or ammmoniacal silver nitrate followed by fluorescein (for hydrochloride salts). Pyridine bases in alcoholic samples containing resinous/volatile matter are estimated in the filtrate obtained after their treatment with alum at pH 5–6. The amount of pyridine and α-picoline is calculated from calibration graphs obtained by plotting the square of the spot diameter against pyridine and α-picoline concentrations. The method allowed detection and estimation of pyridine bases in denatured alcoholic samples at a concentration as low as 60 mg/L. Average recovery of added pyridine bases was 99.4% by this method.

Pyridine and α-picoline are the major ingredients of commercial pyridine bases obtained from coal tars (1). Because of the obnoxious odor and toxicity of these compounds, the Indian Excise authorities have adopted them as denaturants for preparing ordinary denatured spirit (ODS) in a concentration of 5 mL/L (2) along with a similar concentration of caoutchoucine. ODS and french polish of commerce (generally made by dissolving resins like shellac, rosinin ODS) are widely misused for making cheap, illicit potable liquor after removal of their excipients (resins/denaturants) by dilution with water and treatment with alum followed by filtration or decantation. However, it is reported that if pyridine bases are retained as a component of ODS-denaturant, it is not possible to completely remove the denaturant by the above method (3). Bone charcoal recycling has been used effectively for removal of pyridine bases, but it is not economical (4).

Various methods based on thin layer chromatography (5–7), paper electrophoresis (8), spectrophotometry (4, 9), fluorimetry (10), and gas chromatography (11) have been reported for the detection/determination of pyridine bases. Although these methods are sensitive, either there are some obvious limitations to their use in forensic routine work or they involve elaborate instrumental assay. In the present communication we report a simple paper chromatographic method for identification and determination of pyridine bases in ODS french polish and their derived illicit liquors.

METHOD

Apparatus and Reagents

(a) *Chromatographic paper.*—Whatman filter paper No. 3, 12 × 12 cm.

(b) *Spotting capillary pipet.*—10 μL.

(c) *Ultraviolet (UV) viewing cabinet.*

(d) *Solvents.*—Analytical grade methanol, *n*-butanol, acetic acid, ethanol (95%), and ethyl acetate.

Preparation of Standard Solutions

Dissolve 1 g each of accurately weighed pyridine and α-picoline in water in 100 mL volumetric flask. Dilute to volume and transfer 50 mL to an evaporating dish; add 4 mL 6N H_2SO_4 (or 3 mL 4N HCl). Evaporate solution to dryness on water bath. Cool, and dissolve residue in 100 mL 95% ethanol in standard volumetric flask. Prepare series of working standards in 95% ethanol to give desired concentrations.

Denatured Alcoholic Samples

Different commercial samples of ordinary denatured spirit and french polish, all containing denaturants other than pyridine bases, were fortified with different concentrations of pyridine and α-picoline in the range of 0 to 0.8 g/L each. A few illicit liquor samples containing pyridine bases were received from the police authority.

Preparation of Samples

(a) *Ordinary denatured spirit (with and without water-miscible denaturants like methanol.*—To 25 mL ODS (pipet enough sample to supply about 25 mg each pyridine base), add 0.2 mL 6N H_2SO_4 (or 0.3 mL 5N HCl) in evaporating dish. Evaporate mixture to near dryness on water bath. Cool, and dissolve residue in 5 mL 95% ethanol. Retain solution in stoppered flask for analysis.

(b) *Ordinary denatured spirit (with water immiscible denaturants like caoutchoucine).*—To 50 mL sample, containing ca 50 mg each pyridine base, in 100 mL volumetric flask, add 25 mL water, 2 mL 5% potash alum, and 1 mL 2% NH_4OH. Adjust to pH 5–6. Shake the solution and dilute to volume with water. Let precipitate settle (12). Filter and proceed as described under (a), beginning "To 25 mL ODS . . . ."

(c) *French polish (containing resin up to 10% w/v).*—To 25 mL sample (containing ca 50 mg each pyridine base) in 100 mL volumetric flask, add 50 mL water, 10 mL 5% potash alum, followed by 5 mL 2% NH_4OH (adjust pH to about 5–6). Shake the solution and dilute to volume with water. Let precipitate settle (12). (If resin content is more than 10% w/v, increase proportions of alum and NH_4OH to obtain clear filtrate.) Filter, and proceed as described under (a), beginning "To 25 mL ODS. . . . "

(d) *Illicit liquor derived from denatured spirit/french polish.*—To 50 mL sample (filter if necessary) containing ca 3 mg each pyridine base, add 0.05 mL 6N H_2SO_4 (or 0.1 mL 5N HCl) in evaporating dish and evaporate to dryness on water bath. From residue, prepare sample solution as described under (a).

Paper Chromatography

Lightly mark 2 lines with pencil (vertical and horizontal) 1.5 cm from one corner of 12 × 12 cm Whatman No. 3 paper sheet. Mark another vertical line intercepting baseline ca 6 cm from same corner of sheet. Moisten paper sheet in mixture of ca 10 mL HCl and HNO_3 (ca 10% v/v of each) in unsaturated tank until acid front reaches top (to remove impurities). Take out strip, wash with water until it is free from chloride

Table 1. Estimation of pyridine bases and their R_f values obtained by sulfate and hydrochloride salt methods

Sample	Method[a]	Denaturants[b]							
		Pyridine R_f: Direction		α-Picoline R_f: Direction		Pyridine, g/L	α-Picoline, g/L	Unreacted acid R_f: Direction	
		I	II	I	II			I	II
Ordinary denatured spirit[c]	SM	0.24 ± 0.018	0.27 ± 0.019	0.39 ± 0.020	0.33 ± 0.024	1.80 ± 0.303	3.11 ± 0.351	0.60 ± 0.045	0.39 ± 0.019
	HM	0.21 ± 0.017	0.23 ± 0.018	0.39 ± 0.016	0.41 ± 0.047	1.75 ± 0.282	3.15 ± 0.242	—[a]	—[a]
French polish[d]	SM	0.24 ± 0.021	0.27 ± 0.012	0.39 ± 0.025	0.33 ± 0.026	1.30 ± 0.322	2.40 ± 0.221	0.60 ± 0.035	0.39 ± 0.022
	HM	0.21 ± 0.019	0.23 ± 0.015	0.39 ± 0.025	0.41 ± 0.019	1.40 ± 0.301	2.50 ± 0.308	—[a]	—[a]
Derived liquor[e]	SM	0.24 ± 0.020	0.27 ± 0.018	0.39 ± 0.028	0.33 ± 0.025	0.20 ± 0.014	0.36 ± 0.019	0.60 ± 0.043	0.39 ± 0.051
	HM	0.21 ± 0.017	0.23 ± 0.021	0.39 0.019	0.41 ± 0.018	0.25 ± 0.017	0.40 ± 0.016	—[a]	—[a]

[a] SM = sulfate salt method; HM = hydrochloride salt method; ND = not detected.
[b] Mean ± SD for 10 determinations.
[c] Sample containing other denaturants, such as caoutchoucine.
[d] Sample with up to 10% w/v resins.
[e] Sample containing 10 ± 2% v/v ethyl alcohol.

and nitrate ions, and dry with air blower. Use this strip for chromatography a or b below.

(a) *Sulfate salt method.*—Apply aliquots of 10 μL of both standard and sample solution preparations to paper sheet with micro pipet, at marked diagonal points. After air-drying, develop chromatogram in presaturated chamber up to 10 cm in solvent system methanol–butanol–acetic acid–ethyl acetate (20 + 10 + 1 + 10) in first direction. Remove sheet from chamber, dry with cold air, and develop at right angles to the first direction, up to 10 cm in same solvent mixture. Remove strip and dry in cold air. Spray uniformly with spray reagent: freshly prepared 0.2% sodium rhodizonate solution followed by 0.2% barium chloride solution. White spots will appear, due to sulfate salts of pyridine bases, against red-brown background (Table 1). Measure diameter of spots immediately in 2 perpendicular directions, using sliding calliper. Repeat for duplicate sample, and take mean of 4 values (in mm). To obtain calibration curve (13), plot concentration of standard pyridine base applied in μg/10 μL (3–50 μg each) against corresponding diameter in sq. mm.

(b) *Hydrochloride salt method.*—Follow above procedure, with some exceptions. Prepare hydrochloride salts of standard pyridine bases and sample solution as described above under preparation of standard and sample solutions, respectively. Develop chromatogram as described above and dry strip to remove acetic acid as complete as possible. Spray it uniformly with freshly prepared reagent: 1% ammoniacal silver nitrate followed by 0.1% fluorescein solution in ethanol. Expose strip to UV light (5–10 min) in UV cabinet. Grey spots will appear, due to chlorides salts of pyridine bases, against yellow background which changes to orange (Table 1).

The pyridine and α-picoline contents in 3 different types of confiscated samples were estimated by the described methods (a) and (b). The results of assay are tabulated in Table 1.

Results and Discussion

One-dimensional paper chromatography using HCl–HNO₃, pre-washed Whatman papers does not resolve either the sulfate or hydrochloride salts. For sulfate salts particularly, the excess sulfuric acid gives a white trailing background after spraying, and it is difficult to measure the exact area of the resolved component. Development of the chromatogram in the second direction not only diffuses and dilutes excess sulfuric acid, but separates the components distinctly as compact, round spots even at 3 μg concentration, and gives a round spot of unreacted excess sulfuric acid at R_f 0.60 and 0.39 in the first and second directions, respectively.

Pyridine and α-picoline sulfates are located as white spots on a red-brown background by spraying the developed chromatogram with sodium rhodizonate followed by barium chloride solution. The white spots are caused by the formation of insoluble barium sulfate (14). The hydrochlorides of pyridine bases are detected by spraying the chromatogram with ammoniacal silver nitrate followed by fluorescein solution in ethanol (15); the spots are gray in daylight and fluoresce under UV light. No interferences were noted in the hydrochloride salt method. Experimental data obtained showed that both the sulfate and hydrochloride salt methods are equally sensitive (Table 1). We used the hydrochloride salt method for additional confirmation, although either method is acceptable.

The best resolution and most prominent white spots (sulfate) are obtained from pyridine bases ranging from 3 to 50 μg in the aliquot spotted (10 μL) from the prepared 5 mL sample solution that was derived from 25 mL sample. Thus, considering 3 μg the minimum amount detected, we can detect and estimate pyridine bases in the sample at a concentration as low as 60 mg/L. The linear relationship obtained by plotting square of spot diameter versus component concentration is used for the assay.

Commercial samples of ordinary denatured spirit, french polish, and liquor derived therefrom were spiked with pyridine and α-picoline, added separately, at different levels, and analyzed by the described sulfate salt method (a). Table 2 gives recoveries of pyridine and α-picoline from these synthetic mixtures. Recoveries ranged from 95.8% in french polish to 105.3% in liquor derived from ODS. Average recovery of added pyridine bases was 99.4% by the sulfate salt method.

Table 2. Recoveries of pyridine and α-picoline from several synthetic mixtures by sulfate salt method[a]

Sample	Pyridine			α-Picoline		
	Added, mg/L	Found, mg/L	Rec., %	Added, mg/L	Found, mg/L	Rec., %
Ordinary denatured spirit[b]	0	0	—	0	0	—
	300	298.6	99.5	300	299.4	99.8
	400	402.4	100.6	400	404.4	101.1
Ordinary denatured spirit[c]	0	0	—	0	0	—
	300	294.8	98.3	300	295.6	98.5
	400	395.2	98.8	400	392.8	98.2
French polish[d]	0	0	—	0	0	—
	400	390.5	97.6	500	480.6	96.1
	800	770.8	96.3	700	670.8	95.8
Liquor derived from (b)	0	0	—	0	0	—
	200	210.6	105.3	200	206.0	103.0
	300	298.4	99.5	300	294.8	98.2
Liquor derived from (c)	0	0	—	0	0	—
	200	205.6	102.8	200	202.8	101.4
	300	295.6	98.5	300	203.4	97.8

[a]All values are averages of duplicate determinations.
[b]Commercial samples initially containing denaturants (like methanol) other than pyridine bases.
[c]Commercial samples initially containing denaturants (like caoutchoucine) other than pyridine bases.
[d]Commercial samples initially containing denaturants (like methanol/caoutchouchine) other than pyridine bases with up to 10% w/v resinous matter.

REFERENCES

(1) Davis, W. A., & Sodtler, S. S. (1912) *Allen's Commercial Organic Analysis*, Vol. VI, J & A Churchill, London, UK, pp. 140–143
(2) I.S.I. Specification for Alcohol Denaturants, IS NO: 4117 (1966) p.14
(3) Pai, P. P., Vaidya, P. V., Joshi, S. B., & Ambade, K. A. (1974) *J. Indian Acad. Forensic Sci.* 13, 10–12
(4) Kulkarni, J. P., Pai, P. P., & Mattoo, B. N. (1978) *J. Indian Acad. Forensic Sci.* 17, 73–75
(5) Jakovlievic, I. M., Bishara, R. H., & Kress, T. J. (1977) *J. Chromatogr.* 134, 238–241
(6) Damani, L. A., Patterson, L. H., & Gorrod, J. W. (1978) *J. Chromatogr.* 155, 377–348
(7) Pearse, G. A. J., & Ericsson, M. (1979) *J. Chromatogr.* 177, 174–176
(8) Wagner, H., & Lehmann, H. (1977) *Anal. Chem.* 283, 115–119
(9) Rawat, J. P., & Singh, J. P. (1977) *Science* 12, 281–288
(10) Wong, M. P., & Connors, K. A. (1978) *Anal. Chem.* 59, 2051–2054
(11) Wood, G. O., & Anderson, R. G. (1976) Report of United States Energy Research Development Administration, LA-6216-PR, p.13
(12) Ambade, K. A., & Maghal, S. K. (1979) *Research and Industry* 24, 38–41
(13) Hulthe, P. (1970) *Analyst* 95, 351–355
(14) Feigl, F. (1958) *Spot Tests in Inorganic Analysis*, 5th Ed., Elsevier Publishing Co., Amsterdam, Netherlands, p. 313–314
(15) Thielemann, H. (1976) *Z. Chemic. LPZ.* 16, 283

FOOD COMPOSITION

Gas Chromatographic Determination of Oxalic Acid in Foods

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A new quantitative gas chromatographic (GC) method has been developed for the determination of oxalic acid in foods. Solid sample is extracted with water (soluble oxalic acid) or 2N hydrochloric acid (total oxalic acid) at room temperature. An aliquot of sample extract is evaporated to dryness, and the oxalic acid in the residue is methylated with 7% hydrochloric acid–methanol. The reaction mixture is extracted with chloroform, and dimethyl oxalate is quantitated by GC. Recovery of oxalic acid added to liquid samples averaged 100.6%; recoveries from extracts of solid samples were 96.2–99.5 and 97.2–100.1% for water and hydrochloric acid extractions, respectively. Results are shown for determination of oxalic acid in spinach and beverages. The technique is simple, rapid, and accurate, and small samples may be used. The limit of determination is 20 μg.

Urolithiasis is one of the most common diseases in urology, and over 70% (1) of the urinary calculi consist of calcium oxalate. Oxalic acid in urine is derived from diet and endogenous origins such as ascorbic acid, glycine, glycolates, etc. Up to 50% of urinary oxalate may be derived from dietary oxalate on a low calcium diet, nearly 15% for a healthy person on a well balanced diet (2). This suggests that dietary oxalate plays an important role in the formation of calculi.

The determination of oxalic acid in foods as well as in urine and blood is essential for the investigation of urolithiasis and related subjects. Permanganate (3–7), colorimetric (8–11), and conductometric titration (12) methods have been used to determine oxalic acid in foods; however, these methods are complicated, time consuming, and sometimes inaccurate. Recently, the determination of oxalic acid in foods was reported by enzymatic (13) and liquid chromatographic (14) methods. There are several gas chromatographic (GC) techniques (15–17) for determining plant organic acids, including oxalic acid. The few references (18) found for GC analysis of oxalic acid in biological materials offer unsatisfactory chromatograms.

The author and 2 colleagues recently reported a simple, rapid, accurate GC method (19) for the determination of urinary oxalate. The present paper describes application of that GC method, with several modifications, to foods, and includes a new procedure for extracting oxalic acid from solid samples.

METHOD

Apparatus

(a) *Homogenizer.*—Nihon Seiki Model HA II equipped with 200 mL glass cup (Nihon Seiki Co., Ltd, Tokyo, Japan).

(b) *Centrifuge.*—Marusan Model 55-1 (K. K. Sakuma Seisakusho, Ltd, Tokyo, Japan).

(c) *Vertical wrist-action shaker.*—KM Universal shaker Model V-S (Iwaki Co., Ltd, Tokyo, Japan).

(d) *Gas chromatograph.*—Yanaco Model G 80 equipped with FID detector (Yanagimoto Manufacturing Co., Ltd, Kyoto, Japan).

(e) *Column.*—2.25 m × 3 mm id glass packed with Unisole F 200 (Gasukuro Kogyo Co., Ltd, Tokyo, Japan).

Reagents

(a) *Hydrochloric acid.*—2N (concentrated HCl–water, 1 + 5).

(b) *Methylating reagent.*—7% HCl–methanol (concentrated HCl–methanol, 1 + 4).

(c) *Internal standard (IS) solutions.*—IS solution I—2 μL methyl caprylate/mL methanol. *IS solution II*—10 μL methyl caprylate/mL methanol.

(d) *Oxalic acid standard stock solution.*—5 mg (as $C_2H_2O_4$)/mL water. Use guaranteed reagent oxalic acid ($C_2H_2O_4.2H_2O$) and keep in refrigerator. Prepare standard solution of 0.5 mg/mL or 0.1 mg/mL from stock solution just before use.

Extraction

Cut solid sample into pieces. Weigh pieces and homogenize 5 min with same weight of water.

(a) *Soluble oxalic acid.*—Transfer 10 g slurry (5 g sample) to glass blender cup with 20 mL water and blend 5 min at highest speed. Transfer mixture to two 50 mL centrifuge tubes and centrifuge 5 min at 10000 rpm. Transfer supernate to 100 mL Erlenmeyer flask. Return remainder to blender cup and repeat extraction twice more. Collect supernate in same flask and add HCl to adjust pH to 2 (20). Transfer extract to 100 mL volumetric flask and dilute to volume with water.

(b) *Total oxalic acid.*—Extract 10 g slurry 3 times in same manner as above, except use three 20 mL portions of 2N HCl. Collect supernates in 100 mL volumetric flask and dilute to volume with water.

Add HCl to liquid sample to adjust pH to 2 (20), and measure volume.

Methylesterification

Pipet aliquot of extract containing 0–500 μg oxalic acid into 10 mL test tube or round-bottom flask of suitable volume, and evaporate to dryness by using vacuum rotary evaporator in 40–45°C water bath. Add 5 mL 7% HCl–methanol and appropriate volume of IS solution: 10 μL IS solution I (0–100 μg oxalic acid) or 20 μL IS solution II (100–500 μg oxalic acid). Stopper test tube or flask, and place in 60°C water bath for 30 min.

Chloroform Extraction

Transfer reaction mixture to 50 mL separatory funnel. Add 1 mL $CHCl_3$ and 10 mL water, and shake 15 min. Transfer lower layer into test tube with stopper. Prepare three $CHCl_3$ extracts from a sample (or sample extract).

Gas Chromatography

Use a 10 μL aliquot of $CHCl_3$ solution for chromatography. Operating conditions: column 80°C (0–100 μg oxalic acid) or 90°C (100–500 μg); injection port 150°C; N carrier gas 20 mL min; sensitivity 10^9 Ω; attenuator 1/2(0–100 μg oxalic acid) or 1/16(100–500 μg); chart speed 5 mm/min.

Analyze each $CHCl_3$ solution 3 times and measure peak heights of dimethyl oxalate and IS. Determine amount of
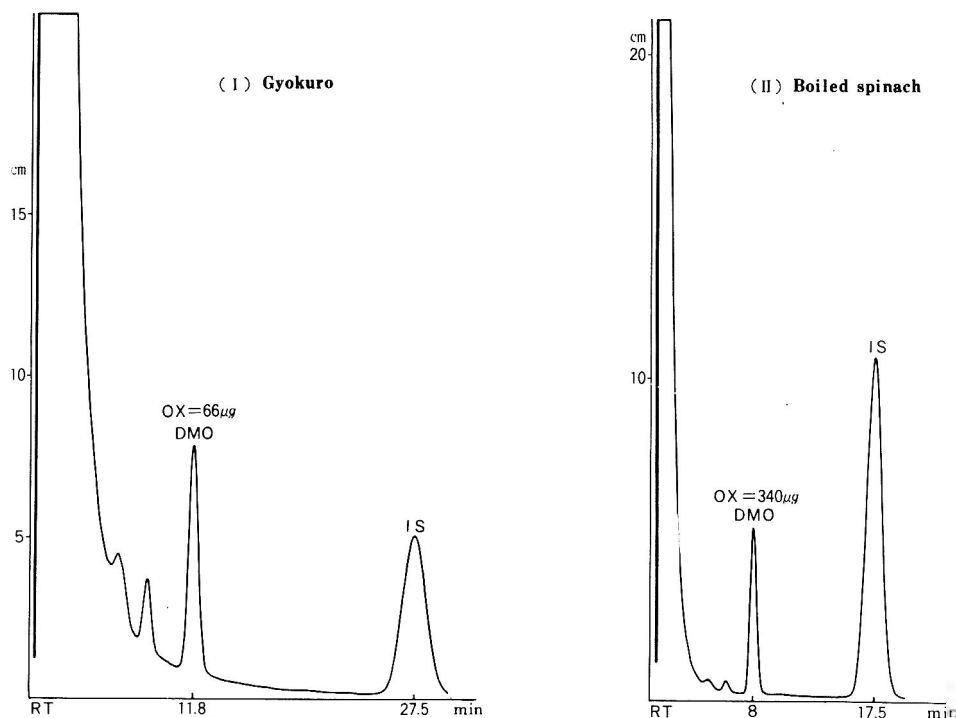
Figure 1.   Chromatograms of oxalic acid in tea (I) and spinach (II). Peaks are dimethyl oxalate (DMO) and methyl caprylate (IS). See text for chromatographic conditions.

oxalic acid from corresponding calibration curve, and calculate oxalic acid content of sample.

Preparation of Calibration Curves

(a) *Calibration curve I (0–100 μg oxalic acid).*—Add various volumes of 0.1 mg/mL standard solution to six 10 mL test tubes to prepare a series of oxalic acid standard solutions (0, 20, 40, 60, 80. and 100 μg). Fill all tubes, except the 100 μg standard, with water to volume of 1 mL and evaporate to dryness with a vacuum rotary evaporator at 40–45°C. Then add 10 μL IS solution I and 5 mL 7% HCl–methanol to each tube. After 30 min at 60°C, transfer reaction mixtures to separate 50 mL separatory funnels and add 1 mL $CHCl_3$ and 10 mL water to each. Shake 15 min, then draw off lower layer into a test tube with stopper. Use a 10 μL aliquot for GC analysis. Plot calibration curve I from peak height ratios of dimethyl oxalate to IS. Curve is almost linear up to 100 μg, with the formula $Y = 0.0200X - 0.0438$ ($r = 0.999$, $N = 18$, Y = peak height ratio, X = oxalic acid content (0–100 μg)).

(b) *Calibration curve II (100–500 μg oxalic acid).*—Prepare curve II according to the procedure for curve I, but use 0.5 mg/mL standard solution and 20 μL IS Solution II. The equation for the relationship was $Y = 0.0015 - 0.0060$ ($r = 0.999$, $N = 18$, Y = peak height ratio, X = oxalic acid content (0–500 μg)).

Results

Extraction from solid sample.—Ten g portions of different boiled spinach slurries (5 g sample) were extracted with each of 20 mL water or 2N HCl according to the procedure. The first, second, and third extracts contained 7.39 mg (76.4%), 1.53 mg (15.8%). and 0.75 mg (7.8%) oxalic acid for water extraction, and 31.50 mg (87.2%), 3.93 mg (10.9%), and 0.68 mg (1.9%) for 2N HCl extraction, respectively. The chromatographic peak heights of dimethyl oxalate in the third extracts with water and 2N HCl were 0.17 and 0.30 cm, respectively. Therefore, we concluded that three extractions are sufficient for extraction of oxalic acid in solid sample.

Ten g boiled spinach slurry (5 g sample) was extracted with three 20 mL portions of various concentrations of HCl. The quantity of total oxalic acid extracted at room temperature was constant between 0.5 and 3N HCl. In addition, warming during the extraction procedure as recommended in some references (6, 11) proved to be unnecessary. We selected 2N as the optimum HCl concentration for extraction of total oxalic acid in solid sample.

Time for esterification.—The degree of methyl esterification of oxalic acid with 7% HCl-methanol was examined several times during 60 min following addition of the reagent. Oxalic acid was methylated almost instantaneously. Thirty min was selected as the esterification time.

Stability of $CHCl_3$ solution.—Peak height ratios for the $CHCl_3$ extracts (100 and 500 μg oxalic acid) did not change significantly over 12 days. GC analysis can be delayed for several days after extraction.

Chromatography.—Figure 1 shows gas chromatograms of dimethyl oxalate and IS which were obtained for analysis of tea and boiled spinach samples. The dimethyl oxalate peaks are sharp and sufficient in height for determination.

Recovery tests.—Recovery of oxalic acid from liquid samples was studied by adding 200 and 400 μg oxalic acid to 1 mL boiled water in which spinach was cooked. The average recovery was 100.6% ± 2.0 (SD) (Table 1). Recovery from solid samples was determined by adding oxalic acid to raw cabbage slurry and boiled spinach slurry. Samples were extracted with water or 2N HCl. Recoveries were between 96 and 100% for water extraction and 97 and 100% for 2N HCl extraction (Table 2).

Determination of oxalic acid in spinach and beverages.—About 100 g raw spinach was boiled 1 min in 1 L water and was soaked in 1 L tap water. After 1 min, the boiled spinach was squeezed. Data in Table 3 show that nearly 30% of the oxalic acid in raw spinach is removed by boiling. In raw spinach, 48% of the oxalic acid is insoluble; the remainder is soluble, of which about half is retained in boiled spinach.

Table 1. Recovery of oxalic acid added to boiling water from spinach

Sample	Oxalic acid		
	Added, μg	Found,[a] μg	Rec., %
1	0	120.63	—
	200	326.88	103.1
	400	526.25	101.4
2	0	118.13	—
	200	315.63	98.8
	400	515.00	99.2
		Av. ± SD	100.6 ± 2.0

[a]Average of 3 determinations

Table 2. Recovery of oxalic acid added to raw cabbage and boiled spinach slurries

Sample, extn soln	N	Oxalic acid		
		Added, mg	Found, mg	Rec., % ± SD
Raw cabbage		0	0	—
Water	9	2.5	2.40	96.2 ± 1.7
2N HCl	9	10	9.72	97.2 ± 1.7
Boiled spinach				
Water	6	0	9.57	—
	6	5	14.54	99.5
Water	6	0	19.08	—
	6	5	23.95	97.4
Water	9	0	18.74	—
	9	10	28.50	97.6
			Av.	98.2 ± 1.2
2N HCl	9	0	32.99	—
	9	10	42.99	100.1
	9	20	52.85	99.3
			Av.	99.7 ± 0.6

Two g tea or coffee sample was infused or dissolved as shown in Table 4, and the oxalic content was determined. Gyokuro, the highest grade green tea, contained more oxalic acid than did black tea.

Discussion

This method can be performed in an ordinary chemical laboratory equipped with common apparatus, including a gas chromatograph with a flame ionization detector. The simple procedure can be performed by any experienced analyst.

After testing several packing materials suggested in the references, we selected a glass column packed with Unisole F 200, which provided the best chromatogram of dimethyl oxalate.

About 2 h is required to prepare the water or 2N HCl extract from solid sample, and 30 min is required for infusion of tea leaves. For extract or liquid sample containing >100 and <100 μg oxalic acid, 2.5 and 3 h, respectively, are required for the determination procedure, consisting of evaporation to

Table 3. Determination of oxalic acid in spinach

Sample	Oxalic acid, mg/100 g raw sample[a]
Boiled spinach water	75.8 ± 2.6
Soaked spinach water	112.1 ± 6.4
Boiled spinach	162.5 ± 2.6 (sol.)
	444.1 ± 1.9 (total)
Raw spinach	344.4 ± 2.3 (sol.)
	657.1 ± 6.9 (total)

[a]Average of 9 determinations ± SD.

dryness, methylation, extraction, and 3 GC analyses. Thus, the entire procedure can be completed within 3 to 5 h.

The determination limit was considered to be 20 μg from the peak height (1.4 cm) of the dimethyl oxalate curve, which corresponded to 2 mg oxalic acid/100 mL liquid sample and 40 mg oxalic acid/100 g solid sample. The limits can be reduced to 0.2 mg/100 mL and 4 mg/100 g, respectively, by using 10 mL liquid sample or extract from solid sample. Only 1 mL extract is sufficient for analysis of foods, such as spinach, which are high in oxalic acid.

Recoveries of oxalic acid in liquid sample are satisfactory, although recovery rates in solid sample with water extraction are scattered. This point must be investigated.

Zarembski and Hodgkinson (9) reported that carbohydrates could be degraded to oxalic acid by heating with 30% HCl in a boiling water bath for 5 h. Our experiment confirmed that 65.0 mg oxalic acid was produced from 100 g potato starch by heating with 35% HCl under the same conditions, but no conversions were observed after extraction with 2N HCl at room temperature. The possibility of oxalogenesis (21) from other progenitors during the procedure should be carefully examined, however.

REFERENCES

(1) Yoshida, O. (1979) *Jpn. J. Urol.* **70**, 975–983
(2) Hodgkinson, A. (1976) *Scientific Foundations of Urology*, Vol. I, William Heinemann Medical Books Ltd, London, UK, p. 291
(3) Kohman, E. F. (1939) *J. Nutr.* **18**, 233–246
(4) Andrews, J. C., & Viser, E. T. (1951) *Food Res.* **16**, 306–312
(5) Baker, C. J. L. (1952) *Analyst* **77**, 340–344
(6) Kusama, M., Murakami, H., & Kidokoro, Y. (1963) *Jpn. J. Nutr.* **21**, 41–45
(7) Kusama, M., Murakami, H., & Kidokoro, Y. (1964) *Jpn. J. Nutr.* **22**, 23–26
(8) Yamazoe, Y. (1959) *Kagaku (Tokyo)* **29**, 468
(9) Zarembski, P. M., & Hodgkinson, A. (1962) *Analyst* **87**, 698–702
(10) Zarembski, P. M., & Hodgkinson, A. (1962) *Br. J. Nutr.* **16**, 627–634

Table 4. Determination of oxalic acid in tea and instant coffee

Sample	Oxalic acid, mg[a]	Sample Treatment		
		Water vol., mL	Temp., °C	Infusion
Green tea				
Gyokuro, highest grade	13.09 ± 0.27	50	57	3 min
Sencha, common grade	6.81 ± 0.20	60	75	90 s
Bancha, coarse grade	4.36 ± 0.21	60	95	instantaneous
Black tea				
Tea bag	11.07 ± 0.27	180	boiling	1 min
Darjeeling tea	9.18 ± 0.24	180	boiling	1 min
Blended tea	9.18 ± 0.26	180	boiling	1 min
Instant coffee	1.04 ± 0.05	120	85	—

[a]Average of 9 determinations ± SD; 2 g samples.

(11) Nakahara, K. (1974) *J. Jpn. Soc. Food Nutr.* **27,** 33–38
(12) Goto, E., & Matunaga, A. (1978) *J. Home Econ. (Tokyo)* **29,** 567–570
(13) Yamanaka, H., Kuno, M., Shiomi, K., & Kikuchi, T. (1983) *J. Food Hyg. Soc. Jpn.* **24,** 454–458
(14) Wilson, C. W., III, Shaw, P. E., & Knight, R. J., Jr (1982) *J. Agric. Food Chem.* **30,** 1106–1108
(15) Sarkar, S. K., & Malhotra, S. S. (1979) *J. Chromatogr.* **171,** 227–232
(16) Steenkamp, J., & De Villiers, O. T. (1980) *S. Afr. J. Sci.* **76,** 42–43

(17) Janauer, G. A., & Englmaier, P. (1980) *Anal. Biochem.* **107,** 136–139
(18) Mee, J. M. L., & Stanley, R. W. (1973) *J. Chromatogr.* **76,** 242–243
(19) Yanagawa, M., Ohkawa, H., & Tada, S. (1983) *J. Urol.* **129,** 1163–1165
(20) Robertson, W. G., & Rutherford, A. (1980) *Scand. J. Urol. Nephrol., Suppl.,* Vol. 53, No. Proc. Symp. Urolithiasis, 1979, pp. 80, 95
(21) Tocco, D. J., Duncan, A. E. W., Noll, R. M., & Duggan, D. E. (1979) *Anal. Biochem.* **94,** 470–476

METHOD PERFORMANCE

Performance Characteristics of Methods of Analysis Used for Regulatory Purposes. I. Drug Dosage Forms. C. Automated Methods[1]

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For analysis of drug dosage forms, precision measures of AOAC approved automated methods, usually containing a spectrophotometric or fluorometric measurement step, were recalculated on a consistent statistical basis, using a computer program "FDACHEMIST." Ten collaborative studies of 14 compounds in 38 materials, consisting of various dosage forms, usually in 10 replications by an average of 7 laboratories, with a total of 2461 determinations, were reviewed. The average relative standard deviations within-laboratory (RSD_o) and among-laboratories (RSD_x) were 1.1 and 1.9%, respectively, and the ratio of RSD_o/RSD_x was 0.57, with an average outlier rate of 0.57% of the reported values. The line of best fit for RSD_x plotted against $-$log concentration increases slightly with decreasing concentration, extending from an RSD_x of about 1.6% at 100% concentration to an RSD_x of 2.2% at 0.1% concentration, a change in RSD_x of about 0.2% for a 10-fold decrease in concentration, independent of analyte and matrix.

The first paper in this series (1) discussed in detail the results from application of a computer program to the calculation of statistical parameters of methods of analysis applied to drug dosage forms analyzed by partition chromatographic separation on diatomaceous silica followed by spectrophotometric measurement. The second paper (2) applied this computer program to collaborative studies of gas chromatographic (GC) methods of analysis of drug dosage forms. The present paper extends this application to the collaborative studies of all automated methods of analysis which have been approved by AOAC through July 1983 for drug dosage forms. All of these studies except for the first one on phenylephrine (3) have been performed by Associate Referees from the Center for Drug Analysis, Food and Drug Administration, St. Louis, MO.

Procedure

For all collaborative studies of the determination of the active ingredient of drug dosage forms by automated methods published in the *Journal of the Association of Official Analytical Chemists* during the period 1971–July 1983 inclusive, values were recalculated on a uniform basis to provide the statistical parameters listed in Table 1. Outliers were flagged by the following statistical tests: Dixon and Grubbs for extreme values, Cochran for excessive variance of replicates (when present), and Youden rank sum for consistent systematic laboratory bias when 5 or more materials were used in a study. The nomenclature and definitions used and a description of the output from the computer program "FDACHEMIST" are given in the first paper (1) of this series. The nomenclature and symbols used in this paper are those recommended by the AOAC Committee on Interlaboratory Collaborative Studies (4). This involves a slight change in terminology of the precision indices from previous papers, primarily

in the use of repeatability relative standard deviation (RSD_o) and reproducibility (including among-laboratories) standard deviation (RSD_x), both expressed in percentages, instead of the corresponding coefficients of variation.

Analyte concentrations were available for all studies, sometimes by consultation with the authors, who provided the data from original sources.

Results

Table 1 gives the recalculated data for 10 studies (2 on anticoagulants) of automated methods applied to 14 compounds in 38 separate materials (dosage forms). The first 3 columns of the table identify the material studied, its source, concentration (if known by formulation), and the collaborative study and AOAC (1984) method references (5). The number of determinations and laboratories in the next 2 columns, respectively, provide the design of the study. The last 2 headings indicate the repeatability (within-laboratory) (RSD_o) and reproducibility (including repeatability) relative standard deviations (RSD_x), calculated with the indicated percent and kind of statistical outliers removed. The relationship between the 2 RSDs is determined from the equation $s_x^2 = s_L^2 + s_o^2$, where the s values are the standard deviations corresponding to the RSDs in the Steiner notation (6). Although the parameters with the subscript "L," indicating among-laboratories (excluding within-) variability, are not given in Table 1, they are printed out by the program. The first row for each material is always the data calculated with no outliers removed (0/0 in the outlier columns). If outliers are flagged by any of the statistical tests, the next row under the material provides the RSDs with the indicated percent and kind of outliers removed. Determination of whether outliers are sequential or alternative to the previous row can be ascertained by comparison of the values under the column "number of determinations" and the column "number of laboratories" with those of the previous rows. The number of the laboratory responsible for the outlier is given in brackets to show consistent laboratory bias, when present. If no outliers are flagged initially, or if no further outliers are indicated on recycling, the next row contains the next dosage form from the same study or, after the last material, from a new study. Each RSD is presented in 2 columns. The values on the right under each heading are the RSDs which were accepted with the indicated percent outliers (often 0); the values on the left are the RSDs which were not accepted because *(1)* they could be improved substantially by removal of the flagged outlier(s), *(2)* outlier removal did not substantially improve the previously calculated RSD_x, or *(3)* the previous values were already acceptable. Figure 1 gives the accepted RSD_x values as a function of $-$ log fractional concentration, but labeled linearly. The individual studies are summarized in Table 2, where the last columns are the simple averages of the accepted RSDs and the associated standard deviations of the averages (shown merely to indicate the dispersion of the RSDs for the materials averaged). Table 3 shows the outliers by number and kind which were eliminated to provide acceptable RSDs.

Table 1. Collaborative study conditions and derived relative standard deviations for determination of various drugs in dosage forms by automated methods. Rows with nonzero values under "Outliers" column are calculated from previous row with indicated outliers omitted. RSDs displaced to right are RSDs accepted with associated percent outliers (often zero)

Compound, JAOAC ref. method ref.	Dosage form	Concn, %	No. of Detns	No. of Labs	Outliers %	Outliers Kind	Parameters RSDo	Parameters RSDx
Phenylephrine 54, 600(1971) 36.147–52(1984)	0.20% com. soln	0.201	55	11	0	0	0.72	1.39
	0.118% syn. soln	0.118	55	11	0	0	0.56	3.08
	0.512% syn. soln	0.561	51	11	0	0	1.29	2.36
		0.560	46	10	10	99C[3]	0.55	2.03
Methenamine 56, 647(1973) 36.250–55(1984)	325 mg tab.	95.6	60	6	0	0	1.51	1.87
		95.7	59	6	2	95DI/C99[6]	1.24	1.66
	97.38% syn.	97.1	60	6	0	0	1.07	1.34
		97.1	58	6	3	C99[3]	0.92	1.26
	500 mg tab.	52.0	60	6	0	0	1.31	2.44
		52.1	59	6	2	C99[6]	1.02	2.33
	325 mg tab.	52.6	60	6	0	0	1.07	1.58
		52.9	50	5	17	D95[3]	1.11	1.17
Na Warfarin 56, 682(1973) 37.143–50(1984)	2.5 mg tab.	1.12	65	7	0	0	1.33	1.60
		1.12	64	7	2	C96[1]	1.20	1.52
	5.0 mg tab.	2.20	65	7	0	0	0.94	2.23
		2.20	63	7	3	C96[4]	0.82	2.05
		2.20	62	7	5	C96[1]	0.75	1.95
	10 mg tab.	4.34	65	7	0	0	0.85	1.71
		4.34	64	7	2	C96[4]	0.76	1.62
Dicumarol	50 mg tab.	32.1	65	7	0	0	1.12	1.59
		32.1	64	7	2	C99[1]	0.96	1.48
		32.1	62	7	5	C99[1]	0.82	1.45
Acenocoumarol 58, 80(1975)	4.0 mg tab.	3.05	70	7	0	0	1.06	2.08
		3.05	69	7	1	C99[3]	0.87	1.85
Phenprocoumon	3.0 mg tab.	1.47	70	7	0	0	2.89	3.61
		1.46	69	7	1	C99/99DI[3]*	0.89	2.72
K Warfarin	5.0 mg tab.	2.16	70	7	0	0	1.24	2.03
Digoxin 58, 70(1975) 38.165–72(1984)	0.125 mg tab.	0.126	60	6	0	0	2.20	3.32
	0.25 mg tab.	0.179	60	6	0	0	0.88	2.86
		0.179	59	6	2	C95	0.83	2.86
	0.50 mg tab.	0.346	60	6	0	0	1.20	1.83
Reserpine 59, 289(1976) 38.119–25(1984)	0.1 mg tab.	0.123	70	7	0	0	1.58	4.05
		0.123	69	7	1	C99[4]	1.48	3.93
		0.123	68	7	3	C99[4]	1.29	3.96
		0.125	60	6	14	95D[5]	1.48	2.34
		0.125	59	6	16	C99[4]	2.22	2.50
		0.125	58	6	17	C99[4]	1.10	2.83
		0.125	57	6	19	99C[4]	0.88	2.09
	0.25 mg tab.	0.155	70	7	0	0	1.11	2.86
		0.155	60	6	14	C99[2]	0.93	3.04
	0.5 mg tab.	0.231	70	7	0	0	2.15	2.84
		0.231	69	7	1	99DI/C99	1.35	2.24
Prednisolone 60, 27(1977) 39.061–67(1984)	2.5 mg tab.	0.258	60	6	0	0	1.30	2.01
		0.257	59	6	2	C99[1]	1.20	1.85
		0.257	57	6	5	C99[1]	1.03	1.63
	5 mg tab.	0.488	60	6	0	0	1.11	1.87
		0.488	59	6	2	C98[6]	0.99	1.78
Prednisone	2.5 mg tab.	0.259	60	6	0	0	1.16	1.90
		0.259	59	6	2	C99[6]	0.96	1.85
		0.259	58	6	3	C99[6]	0.89	1.79
		0.261	48	5	20	95G[2]	0.95	1.16
	5 mg tab.	0.495	60	6	0	0	0.88	1.44
		0.495	58	6	3	C99[6]	0.78	1.39
		0.495	57	6	5	C99[6]	0.73	1.38
		0.495	56	6	7	C99[6]	0.68	1.38
		0.495	55	6	8	C99[6]	0.64	1.39
		0.495	50	5	17	C99[6]	0.60	1.41
Ferrous sulfate 61, 968(1978) 36.078–84(1984)	150 mg com. cap.	30.9	80	8	0	0	1.81	1.96
		30.9	77	8	4	C99[8]	1.37	1.69
		30.9	70	7	13	C99[8]	1.30	1.60
	300 mg com. tab.	51.9	80	8	0	0	1.99	2.40
		52.0	79	8	1	99DI[8]*	1.33	1.98
		51.9	70	7	13	99C[8]	1.06	1.89
		52.1	70	7	13	98D[4]	2.09	2.12
		52.2	69	7	14	99C[8]	1.38	1.48
		52.2	60	6	25	D&C[4&8]	1.08	1.26
		51.9	60	6	25	C&C[8&1]	0.93	1.86
		52.2	50	5	38	[4&8&1]	0.93	1.13
		51.9	50	5	38	[8&1&7]	0.81	1.98
		52.3	40	4	50	[4&8&1&7]	0.77	1.02
	325 mg com. tab.	55.1	80	8	0	0	1.07	2.03
		55.1	79	8	1	99DI/C[8]	0.89	1.93

Table 1. (continued)

Compound, JAOAC ref. method ref.	Dosage form	Concn, %	No. of Detns	No. of Labs	Outliers %	Outliers Kind	RSD$_o$	RSD$_x$
		55.4	70	7	13	95D[4]	1.09	1.40
		55.4	69	7	14	[8&4]	0.89	1.18
	220 mg/mL com. elix.	4.5	40	8	0	0	0.63	2.39
Chlorpheniramine maleate 62, 1197(1979) **36.127–34(1984)**	4 mg com. tab. A	2.03	70	7	0	0	1.12	1.64
	4 mg com. tab. E	2.00	70	7	0	0	0.96	1.25
	4 mg com. tab. F	1.74	70	7	0	0	1.52	1.84
	4 mg com. tab. G	2.04	70	7	0	0	1.17	1.49
		2.04	68	7	3	C99[4]	0.98	1.37
		2.04	60	6	14	C99[4]	0.98	1.43
Nitroglycerin 63, 696(1980) **36.235–42(1984)**	0.6 mg com. tab (No.32)	1.74	60	6	0	0	0.39	1.21
		1.74	59	6	2	C98[5]	0.36	1.16
		1.74	50	5	17	C/R[5]	0.32	1.03
	0.6 mg com. tab. (No.95)	1.78	60	6	0	0	0.42	1.04
		1.77	59	6	2	C98[5]	0.39	0.99
		1.78	50	5	17	C/R[5]	0.35	0.89
	0.4 mg com. tab. (No.68) (blind duplicate)	1.20	60	6	0	0	0.53	0.96
		1.20	50	5	17	R[5]	0.54	0.89
	0.4 mg com. tab. (No.69) (blind duplicate)	1.20	60	6	0	0	2.91	2.91
		1.20	58	6	3	C99/DI99[6]	0.47	0.90
		1.20	50	5	17	C99[6]	0.45	0.92
		1.20	50	5	17	R[5]	3.19	3.19
		1.20	40	4	33	[5]&[6]	0.48	0.88
	0.3 mg com. tab. (No.85)	0.88	60	6	0	0	0.75	1.33
		0.88	59	6	2	C99/DI99[6]	0.36	1.02
		0.89	58	6	3	C99[6]	0.32	0.95
		0.89	56	6	7	C99[6]	0.29	0.88
		0.88	50	5	17	C99[6]	0.27	0.80
		0.88	50	5	17	R[5]	0.82	1.30
		0.88	40	4	33	[5]&[6]	0.28	0.70
	0.3 mg com. tab. (No.27)	0.89	60	6	0	0	0.39	1.02
		0.89	59	6	2	C97[6]	0.36	1.02
		0.89	50	5	17	C97[6]	0.33	1.10
		0.89	50	5	17	R[5]	0.41	0.96
		0.89	40	4	33	[5]&[6]	0.34	1.06

Abbreviations: com. = commercial product of labeled composition
syn. = formulated product of known composition
tab. = tablet
cap. = capsule
elix. = elixir
D = Dixon laboratory average outlier
C = Cochran outlier
DI = Dixon individual value outlier
G = Grubbs outlier
R = Rank sum outlier laboratory

The number associated with the outlier indications is the one-sided confidence level at which the outlier was flagged; *indicates that the value removed is an outlier by the two-tail Dixon test at the overall 95% CL; the number in square brackets is the number of the laboratory responsible for the outlier. It is consistent only within an individual study.

COMBINED DATA
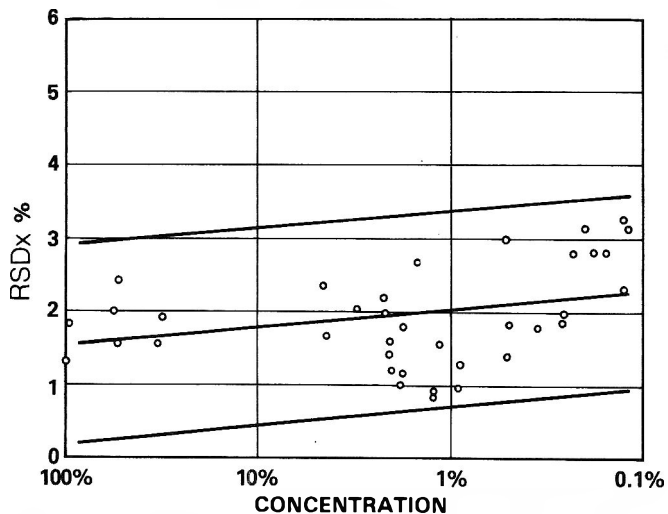
Figure 1. Overall relative standard deviations among-laboratories (RSD$_x$) as function of concentration (spaced logarithmically but labeled in percent) for accepted RSD$_x$ data from collaborative studies for drug dosage forms analyzed by automated methods. Center line is line of best fit which is bracketed by 95% prediction limits.

Discussion

One of the most interesting conclusions of the previous review of the precision of drug methods (7) was that the automated methods used in the 6 collaborative studies examined showed no better precision than did the manual methods reviewed. Four studies have now been added. The statistical parameters of the data from all 10 studies, covering 38 materials (dosage forms), are given in Table 1 and summarized in Table 2. The overall reproducibility (RSD$_x$) of automated methods is now 1.9% as compared with the previous 2.2%; overall repeatability (RSD$_o$), 1.1% vs 1.3%; and the ratio RSD$_o$/RSD$_x$, 0.57 vs 0.59. These parameters are comparable to those of the 2 types of methods previously re-examined (liquid chromatographic and GC). Because of the fairly wide dispersion of the RSDs contributing to the averages, the shift during the past 5 years is not statistically significant. The downward trend with time for both RSDs may reflect an improvement with experience in the preparation of materials for the studies as well as in the analytical work.

Automated methods were previously characterized as exhibiting fewer outliers than other classes of methods. This characteristic is maintained in the current series. Although

Table 2. Summary of statistical parameters of automated methods of analysis applied to drug dosage forms given in Table 1

Analyte or class	No. of		Av. No. of labs	Outliers, %	Relative standard deviations	
	Mater.	Detns			RSD$_o$	RSD$_x$
Phenylephrine	3	161	11	0	0.86 ± 0.38	2.28 ± 0.85
Methenamine	4	240	6	0	1.24 ± 0.21	1.81 ± 0.47
Anticoagulants	7	470	7	0.2	1.06 ± 0.18	1.99 ± 0.41
Digoxin	3	180	6	0	1.43 ± 0.69	2.67 ± 0.76
Reserpine	3	210	7	4.8	1.58 ± 0.53	2.68 ± 0.29
Steroids	4	240	6	0	1.11 ± 0.17	1.81 ± 0.25
Ferrous sulfate	4	320	8	0	1.38 ± 0.64	2.20 ± 0.23
Chlorpheniramine maleate	4	280	7	0	1.19 ± 0.24	1.56 ± 0.25
Nitroglycerin	6	360	6	0.83	0.43 ± 0.06	1.03 ± 0.10
Average or (total)	(38)	(2461)	7	0.57[a]	1.09[b]	1.91[b]

[a]Calculated from a total of 14 values removed from 2461 determinations.
[b]Weighted for number of materials used in each study.

Table 3. Numbers of materials by study for which original data had to be reiterated to obtain "acceptable" or homogenous reproducibility RSD$_x$ because of presence of indicated type of outliers

Analyte or class	None	No. and kind	Total
Phenylephrine	3	0	3
Methenamine	4	0	4
Anticoagulants	6	1 C99/99DI	7
Digoxin	3	0	3
Reserpine	2	1[a] 95D	3
Steroids	4	0	4
Ferrous sulfate	4	0	4
Chlorpheniramine maleate	4	0	4
Nitroglycerin	5	1[b] C99/DI99 1 C99/DI99	6
Totals	34	4	38

[a]One material, but all 10 values from one laboratory removed.
[b]Two values were removed for this material.

at least one material in each of the 10 studies has at least one value flagged as a statistical outlier by one of the 4 tests applied, with their associated confidence levels, outliers had to be removed from only 3 studies (30%) to bring the RSDs into the historical range. Calculated on a number of materials (38) basis, 4 (11%) had to have outliers removed, and calculated on a number of determinations reported (2461) basis, only 14 values (0.6%) had to be eliminated. This value of 0.6% outliers is to be compared with 2.5% of the determinations removed as outliers in collaborative studies of the chromatographic separation/spectrophotometric measurement methods (1) and with 1.4% of the determinations removed as outliers in the GC methods (2).

The statistical design of the collaborative studies of automated methods requires the collaborators to perform 10 (sometimes 5) replicate assays, each often equivalent to a single dose. An outlier, when it occurs, is frequently a single value in the series from a single collaborator, and often the outlier occurs as the first value in a set of replicates.

In one case, it was questioned why the last value in a set of replicates was low. It was discovered that the sequence of values had been reversed on publication and that the outlying last value was actually the first value, consistent with the pattern occurring in most other cases. The explanation for low first values is the use of an insufficient number of standard cups preceding the actual test solution to prevent dilution from wash solutions. Good practice now provides the precaution that, if the first test portion(s) gives a low peak, the solutions should be rerun at the end of the sequence. The last peak would either confirm the low value or would permit a conclusion of insufficient equilibration before the measurement. This example emphasizes the importance of following

up the appearance of outliers in collaborative studies. Retention of excellent records by the Associate Referee permitted explaining the outlier rather than relegating it to the unexplained category; however, unexplained outliers do occur occasionally at other positions in the sequence.

The pattern of a low value at the beginning of a series is easily recognized and easily explained as a procedural error, particularly because the value is usually more than 10 standard deviations from the mean of distribution of values of that laboratory. Reporting such aberrant values without investigation results from operators taking too literally the admonition to report all values, including outliers, in collaborative studies. This instruction is intended to ensure that all normal random variates are included. It is not intended to be the basis for avoidance of an investigation as to the cause of the deviates; on the contrary, it should stimulate investigation. The event and its correction should be reported so that a precaution or a revision in the method can be made to avoid future similar errors by inexperienced operators.

The following are some of the special features of the individual studies in chronological order of publication.

Phenylephrine.—The first collaborative study of an automated method for a drug dosage form was conducted by Margosis (3). The published paper contained only averages and standard deviations of 5 determinations per material per laboratory, but the original data were obtained from the author for recalculation. Two analysts reported 2 series of values for 4 dosage forms and 3 for the fifth. These extra series were arbitrarily calculated as laboratories to provide a more balanced design. This treatment did not significantly affect the parameters. Two materials contained the deliberately added interferences salicylamide and acetaminophen. The presence of these materials resulted in high and low recoveries, respectively, of the analyte, as well as relatively high RSD values. The method was approved with the qualification that it was not applicable in the presence of these interfering drugs. Therefore, the data from the 2 materials containing the interfering drugs are not included in Table 1.

The RSD$_x$ values from the 3 phenylephrine materials are relatively heterogeneous, ranging from 1.4 to 3.1%, but are acceptable in the light of all of the studies. The analytical data on the last material (0.512% synthetic solution) contained a Cochran outlier at the 99% confidence level (CL), whose removal merely reduced RSD$_x$ from 2.36 to 2.03%. The acceptance of the lower RSD$_x$ is not worth the accompanying stigma of 10% outliers, although the RSD$_o$ improved from 1.29 to 0.55%, in line with values from the other 2 materials.

Methenamine.—Four materials were examined by 6 laboratories with 10 replications of each material. The analytical

data for each material had an outlier flagged, each of which could be ignored because the RSD$_x$ ranged from 1.3 to 2.4% at an average concentration of about 75%. The analytical data for 3 materials showed Cochran outliers at the 99% CL as the first or second values in the series, and the fourth material showed a laboratory average flagged by the Dixon test at the 95% CL. All of the outliers came from 2 laboratories. Removal of the values triggering the Cochran test slightly reduced RSD$_x$.

Anticoagulants.—Two studies of the same method were performed by 7 laboratories on 5 compounds, single dosage forms of 4 drugs, and 3 different strength tablets of sodium warfarin. Analytical data for only one of the materials (potassium warfarin) did not show the presence of at least one statistical outlier, all of which were flagged by the Cochran test at the 96% CL or above. For analysis of the 3 mg phenprocoumon tablet, the RSD$_o$ and RSD$_x$ values of all data (no outliers removed) were 2.9 and 3.6%, respectively. Figure 2 shows the original data plotted in ascending order of laboratory means. The single wild result from Laboratory 3 (3.47 mg/tablet) was 20 standard deviations from the mean value (indicated by the right vertical arrow at Laboratory 3) calculated with the wild value included. Simultaneously, the laboratory (No. 1) with the lowest average was flagged as a Dixon outlier at the 98% CL. When the single wild result was removed, the RSD$_o$ and RSD$_x$ decreased to 0.9 and 2.7%, respectively, the mean for Laboratory 3 shifted to the left vertical arrow, and Laboratory 1 no longer was flagged as an outlier! When the wild value (from the laboratory with an average that happened to be the nearest neighbor to the laboratory with the lowest average) was removed, the average of Laboratory 3 was reduced sufficiently so that the numerator of the Dixon statistic was diminished to the point where it no longer produced a critical Dixon value. Because of its extreme deviation, the wild value was removed from the phenprocoumon set, but none of the other flagged outliers were omitted.

Digoxin.—Three materials were examined by 6 laboratories in 10 replications each. The analytical data for only one material showed the presence of an outlier, flagged by the Cochran test at the 95% CL, but because the values from the laboratory flagged as the outlier occurred in the middle of the overall distribution by laboratories, removal of the extreme value did not change the RSD$_x$. The original value was acceptable without outlier removal.

Reserpine.—Three tablets of different strengths were examined by 7 laboratories in 10 replications each. Although Cochran outliers were flagged in the analytical data for all materials, their removal had little effect on RSD$_x$. In the analytical data for the lowest dosage tablet (0.12%), one of the laboratories was flagged as a Dixon outlier at the 95% CL, and none of the individual values from this laboratory overlapped any of the points from the other laboratories. Removal of this laboratory reduced RSD$_x$ from 4.05 (no outliers removed) to 2.34%, with 14% outliers. Other Cochran outliers were still present, and RSD$_x$ could be further reduced to 2.09% with 19% outliers; however, the basic decision was whether to retain the original data with no outliers removed, or to accept the removal of the Dixon laboratory average outlier to attain a more "central" RSD$_x$ value of 2.34%. Because this material is one of the lowest concentrations in the entire series, it could support the highest RSD$_x$. but with the first outlying laboratory removed, the RSD$_x$ lies closer to the other RSD$_x$ values of the group. There are good reasons for either removing or retaining the outlying laboratory. The final decision was to remove the outlying laboratory so that
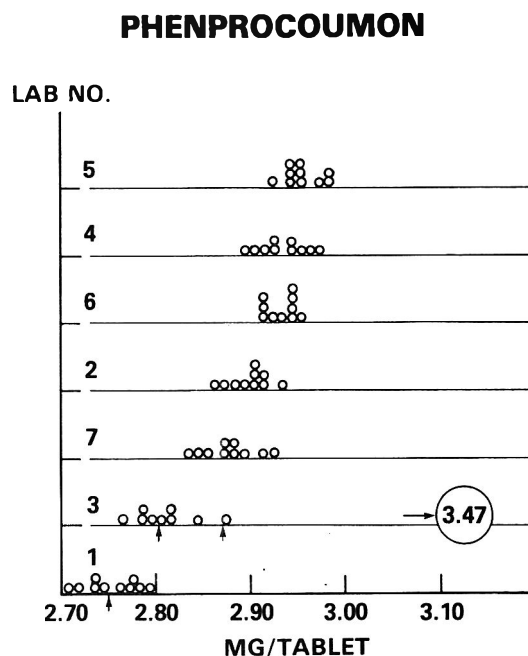
PHENPROCOUMON

Figure 2.   Data from collaborative study of phenprocoumon, concentration in mg/tablet (X-axis) vs frequency (Y-axis) plotted in ascending order of laboratory average concentration. Each circle is a reported value. Vertical arrows for Laboratory 3 are laboratory averages before (right) and after (left) 99+% Cochran outlier (3.47 mg/tablet) is removed.

the cumulative data are more homogeneous, with the penalty of indicating that the RSD$_x$ from this group is accompanied by a 5% outlier rate.

Prednisone and prednisolone.—Two tablets of different strengths of each of these compounds were examined by 6 laboratories in 10 replications each. Numerous sequential Cochran outliers, all from 2 laboratories, were flagged at the 98 and 99% CLs; these were not removed because the original data from each of the 4 materials were satisfactory, and removal of the Cochran outliers in this case would have little effect in reducing RSD$_x$.

Ferrous sulfate.—Four dosage forms—capsules, elixir, and tablets of 2 different strengths—were examined by 8 laboratories in 10 replications each, except for the elixir for which 5 replications were used. The analytical data for all solid dosage forms had flagged statistical outliers; the elixir had no outliers. None of the outliers were removed because the precision parameters as calculated directly were acceptable on historical grounds.

The analytical data from the 300 mg ferrous sulfate tablet were used as an example of a recommended procedure for validation of analytical methods (8). These data, having an acceptable RSD$_x$ of 2.40% with no outliers removed, showed an extensive sequential and simultaneous outlier pattern through 10 iterations. Once outlier removal is begun, there is no logical stopping point until outliers are no longer flagged by the statistical tests used. During this progression, the probability of false rejection increases with each iteration. Figure 3 shows the original data, and Figure 4 diagrams the complex outlier flagging and removal pattern. By consulting both figures simultaneously, in this particular case, the [non]significance of the flags by the various statistical outlier tests can be appreciated in terms of actual values and displacements. Although in this case outlier removal was of no practical significance, following the results of removal of outliers flagged by the various tests led to some very practical conclusions about rules to follow for maximum efficiency (largest improvement in precision with minimum removal of aberrant values).

Figure 3. Original data from 8 laboratories analyzing 10 portions from 300 mg ferrous sulfate tablet composite by automated method. For thes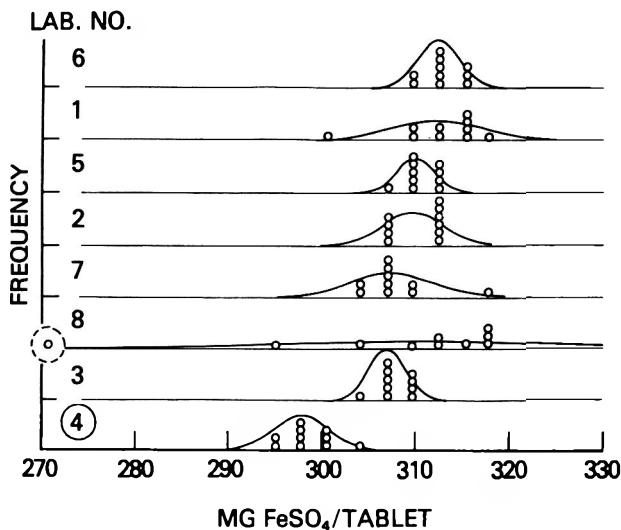e data, concentration in mg/tablet (X-axis) vs frequency (Y-axis) is plotted in ascending order of laboratory average. Each small circle represents reported value for one of the portions. Solid large circle around "4" indicates that Laboratory 4 is Dixon laboratory mean outlier; large dotted circle indicates Dixon individual value outlier (with respect to the other 79 individual values).

In Figure 4, the top box gives the results from the original run of the program for calculating statistical parameters. The first line, "0/0," indicates that no value from any laboratory was removed, and the next line gives the values for RSD_o/RSD_x. In subsequent boxes, the first line may give the nature of the outlier flagged (type and CL) and the laboratory number, in square brackets, activating the flag.

Application of the statistical outlier tests available in the computer program revealed the simultaneous presence of 4 types of statistical outliers in the original data (all of the Dixon tests are $\alpha = 0.05$ one-tail tests applied to both the lowest value and to the highest value in a set):

(a) *Dixon internal laboratory outliers.*—When the Dixon test is applied separately to the 10 values from each laboratory, 5 of the 8 laboratories show the presence of an outlier at CLs of 95–99%. The laboratory numbers exhibiting these outliers, the CL, and an indication as to whether they are high (number to right of "D") or low (number to left of "D") values are given in the box on the extreme right of Figure 4. If these 5 values are removed (6% outlier elimination), RSD_o and RSD_x are 1.17 and 1.97%, respectively, given as the last line in the box.

(b) *Dixon individual value outliers.*—The extremely low value of 270 from Laboratory 8 (given in square brackets) is flagged as a 99% Dixon outlier when tested against all other (79) individual values using an interpolation of the Table C.2 from Thompson (ref. 6, p. 87), which is identical to a Grubbs table. If that one value from one laboratory (indicated by the 1/1 in the second box from the right in the second row) is removed, the RSD_o and RSD_x become 1.33 and 1.98%, respectively, at an outlier rate of 1%. Note the comparability of this RSD_x with that obtained by removing the 6% outliers in the previous case and the significantly lower "stigma" of outlier removal in this case.

(c) *Dixon laboratory average value outliers.*—Laboratory 4 is flagged as a Dixon laboratory average outlier at the 98% CL. Its removal brings RSD_x down to 2.12% at an outlier rate of 13% (extreme left box in Figure 4).

(d) *Cochran outliers.*—Laboratory 8 is also flagged as a Cochran outlier at the 99% CL. The removal of the entire laboratory results in an RSD_x of 1.89% with the stigma of 13% outliers and a reduction of RSD_o from 1.99 to 1.06%. Practically the same reduction in RSD_x is achieved by eliminating the single wild value from this laboratory which was flagged by the Dixon test when it was applied to all individual values. This result suggests 2 points regarding outlier removal, which should be investigated in subsequent studies:

(1) "Wild" results, which are apparent by inspection of the original data or by visual observation of a plot of the data, may be rejected without statistical verification as common sense outliers. To satisfy statistical purists, the probability of the wild value being a member of a normal population of the remaining values may be placed at some arbitrary large number, e.g., 1 in 10^5. Such values can be produced by incorrect recording of irretrievable numbers, such as sample weights. Such values are verifiable as real outliers when they appear in synthetic, formulated, or spiked samples by comparison with the known values. Occasionally the reported value(s) defies the law of conservation of mass.

(2) In removing outliers flagged by the Cochran test from designs containing more than 2 replicates per laboratory, there are 3 ways of proceeding: Remove all values from the flagged laboratory, remove the most extreme value (relative to the overall mean) but retain all other values, or remove both extreme values (one from each end). Occasionally, there may be a cluster of 2 or 3 values at one end, in which case all values from the cluster should be considered for removal as a group. Experience suggests that the most efficient procedure is to remove only the most extreme value(s). The other points are usually "innocent bystanders" whose removal has little effect on the subsequent values of RSD_o and RSD_x. To maintain that 1 or 2 outlying values should trigger the removal of all other values from the same laboratory even though they correspond to the values from other laboratories is not consistent with the accusation made against the rank sum test. The rank sum test, which flags laboratories with consistently low or high ranks over all materials, has been accused of unjustly removing nonoutlying values (1).

Figure 4 also illustrates the noncommutative nature of the application of outlier tests, suggesting that outlier tests should be applied in a systematic order. For example, going down the Cochran laboratory removal path (the center boxes, removing all values from Laboratories 8, 1, and 7 for a total outlier removal of 38%) is considerably less efficient for obtaining a minimum RSD_x than the removal of the single Dixon laboratory average outlier in the removal path at the left of the figure.

All of the outlier tests in this example achieve roughly the same RSD_x values on their first application, as shown in Table 4.

It is seen that for a single "wild" value outlier, the most appropriate application of the outlier tests already in the computer program is the use of the Dixon individual value test. Unfortunately, this test is objectionable on theoretical grounds, because the combined data are not expected to be part of a single normal distribution. The same result may be achieved, however, either by application of the Cochran test so that only the extreme value rather than the entire laboratory is removed, or by simple application of common sense.

Although this discussion leads to some interesting aspects of the results of applying conventional outlier tests, it must not be overlooked that in this case this examination is academic, because the RSD_x was already acceptable without outlier removal.

Chlorpheniramine maleate.—This study is one of the simplest for interpretation of results. Four commercial ground

OUTLIERS

No.	%
0	0
1	1
5	6
10	13
11	14
20	25
30	38
40	50

Flowchart boxes:

- Top center: 0/0   1.99/2.40
- DIXON LABORATORY AVERAGE OUTLIER   DIXON INDIVIDUAL VALUE OUTLIER   DIXON INTERNAL
- 99D[8]   1/1   1.33/1.98
- LAB 1 99D / 3 95D / 5 95D / 7 D99 / 8 95D   1.17/1.97
- COCHRAN RANGE OUTLIER
- 98D [4]   10/1   2.09/2.12
- 99C[8]   10/1   1.06/1.89
- DIXON LAB AVERAGE
- 98D[4]   11/2   1.38/1.48
- COCHRAN   98 DIXON (LAB 4)   COCHRAN   COCHRAN
- [8]   20/2   1.08/1.26
- [1]   20/2   0.93/1.86
- COCHRAN   95 DIXON (LAB 4)   COCHRAN
- [1]   30/3   0.93/1.13
- [7]   30/3   0.81/1.98
- COCHRAN
- [7]   40/4   0.77/1.02

Figure 4. Progression of outlier removal for data given in Figure 3 for 300 mg ferrous sulfate tablets analyzed by 10 laboratories with 10 replicates each. Numbers in box at top center indicate no outliers removed (0/0) and repeatability RSD_o = 1.99% and reproducibility RSD_x = 2.40%. This set of data shows 4 types of outliers (see Figure 3 for interpretation in terms of actual values removed as outliers):

(1) Dixon laboratory average outliers (left path).—Displaced Laboratory 4 is flagged as outlier at 98% CL. If it is removed (10 values from one laboratory indicated by [10/1]), resulting RSD_o and RSD_x values = 2.09 and 2.12%. But now Laboratory 8 is flagged as Cochran outlier, whose removal (now 20 values from 2 laboratories) gives RSD_o = 1.08% and RSD_x = 1.26%. Now Laboratory 1 is flagged as sequential Cochran outlier, etc.

(2) Cochran outliers (center path).—Laboratory 8 has very wide range compared with ranges of other laboratories, much of which is caused by single value flagged in individual value test. When this value is removed, Laboratory 4 is exposed as Dixon laboratory average outlier (98% CL) and Laboratory 1 as Cochran outlier. If Laboratory 4 is removed, RSD_x decreases significantly; if Laboratory 1 is removed, RSD_o and RSD_x are hardly affected. If only Cochran outliers are removed, ultimate RSD_o = 0.81% and ultimate RSD_x = 1.98% (38% outliers removed). However, Laboratory 4 may also be removed after second iteration, joining Dixon laboratory average outlier path.

(3) Dixon individual value outliers.—Extremely low value of 270 from Laboratory 8 is flagged as Dixon individual value outlier (99% CL). If it is removed, (1/1 = 1 value removed from 1 laboratory), resulting values are RSD_o = 1.33% and RSD_x = 1.98%.

(4) Dixon test applied to each laboratory individually.—Applying Dixon test individually to each laboratory, laboratories indicated in box on far right are flagged as containing outliers: 99D indicating lowest value is Dixon outlier at 99% CL; D99 indicates highest value is Dixon outlier at 99% CL; and 95D indicates lowest value is outlier at 95% CL. When these 5 values are omitted, RSD_o = 1.17% and RSD_x = 1.97%, at 6% total outlier rate.

Each of these types of outlier removal has exposed additional outliers, some of which are also flagged by previous iteration. Number in brackets identifies laboratory removed. Iterations stop when no further values are flagged as outliers (when 50% of the data are removed at RSD_o = 0.77% and RSD_x = 1.02%).

Table 4. Comparison of relative standard deviations resulting from removal of outliers by different outlier removal treatments of ferrous sulfate data of Figure 3

	Outliers			
Outlier tests	No.	%	RSD_o	RSD_x
None (original data)	0	0	1.99	2.40
Wild value removal (common sense) Dixon, applied to all individual values Cochran, removing only extreme value	1	1	1.33	1.98
Dixon, applied to each lab. separately	5	6	1.17	1.98
Dixon, applied to lab. averages	10	13	2.09	2.12
Cochran, removing entire lab.	10	13	1.06	1.89

tablets were analyzed by 7 collaborators in 10 replications each. The data appeared to form relatively tight clusters, and only one of the materials displayed a Cochran outlier at the 99% CL. In this case, the laboratory had 2 extreme values, one at each end. When only these 2 values are removed, RSD_x drops from 1.49 to 1.37%; when all values from that laboratory are removed, RSD_x is 1.43%. In either case, the change in RSD_x is not worth the consequence of 3 and 14% outliers, respectively. This example again suggests that removal of only the extreme point(s) from the laboratory flagged by the Cochran test is more efficient than removal of the entire laboratory in dealing with the 10-replicate design of auto-mated methods.

Nitroglycerin.—This study is again interesting from the point of view of the presence of a considerable number of outliers of no consequence, as in the case of the ferrous sulfate dosage forms. Six materials were examined by 6 lab-oratories in 10 replications each. Of greatest significance is the incorporation into the design of blind duplicate materials, designated by the author as Nos. 68 and 69. The study itself exhibits one of the best among-laboratories precisions encountered thus far—about 1.4% when no outliers are rejected, yet it is riddled with statistical outliers, some of which are obvious blunders. Because of this incongruity, the data are plotted in Figure 5 normalized to a 100% label claim. The 10 replicates from each laboratory are plotted as the percent of label claim; the materials are plotted one above the other, and within materials, by laboratory, in ascending order of laboratory average. For each material, the laboratory number is given on the vertical left axis together with the material indentification in terms of mg nitroglycerin/tablet (0.6, 0.4, and 0.3), and, in parentheses, the material identifi-cation number assigned by the author. Although the horizon-tal scale is the same for all materials, its position had to be shifted for several of the materials in order to keep the data on the same graph. Even so, 3 obvious wild outliers could not be included, and are entered as the actual percent of label claim rather than as a point. The short vertical arrows on each horizontal scale are the averages for each material, all data included.

Even before the application of statistical tests for outliers, 2 points are obvious by inspection:

(1) Three values, all from Laboratory 6, in materials 69 and 85 are wild results. To maintain that these 3 values, all from the same laboratory, are part of the population constructed from the other 357 values, when they cannot even appear on the same scale, does not correspond with experience or com-mon sense. The results were recognized as abnormal in the report, which states, "No reason was found for these spu-rious results." When statistical tests are applied, these points are identified as outliers by the Cochran test at more than the 99% CL.

(2) Laboratory 5 always has the highest laboratory average for all 6 materials. This laboratory is flagged as a rank sum outlier by the rank sum test. In this case, its presence does little harm because its laboratory average for most materials is relatively close to that of the next lower laboratory. Lab-oratory 1 has almost always the lowest or near lowest labo-ratory average of the series. When the statistical outlier tests are applied, Laboratory 6 was found to produce a consistent pattern of Cochran outliers with many of the materials, indi-cating a greater range of values than those from the other laboratories.

An important aspect of this study was the presence of identical materials unknown to the collaborators. Material No. 68 showed a very tight pattern of data, with an RSD_o of
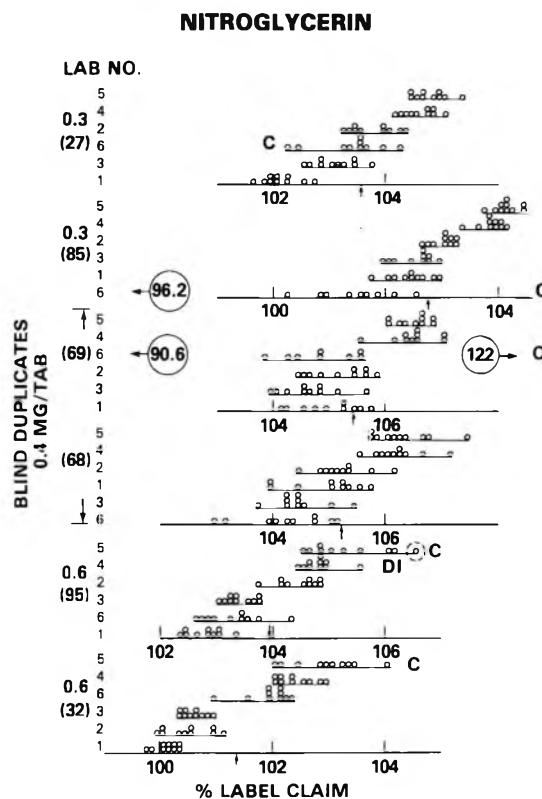
NITROGLYCERIN

Figure 5. Data from collaborative study of nitroglycerin, arranged as in Figure 2. Circled numbers are "wild" values flagged as 99+% Cochran outliers which are removed to obtain "acceptable" statistical parameters. Note particularly the patterns of materials 68 and 69, which are blind duplicates. Patterns are comparable when wild values are removed.

0.53% and an RSD_x of 0.96%. No outliers were flagged, and even when the rank sum outlying Laboratory 5 is removed, the parameters change very little; RSD_o becomes 0.54% and RSD_x becomes 0.89%. The same material run in the same series under a different number, however, showed values of 2.91 and 2.91% for RSD_o and RSD_x, respectively. The fact that RSD_o and RSD_x are the same indicates that the within-laboratory variability swamps out the among-laboratories variability, a situation caused by the presence of the 2 wild values for material 69 from Laboratory 6. When these 2 values are removed, RSD_o and RSD_x become 0.47 and 0.90%, respectively, in line with the values for the other materials in the series. The presence of another wild value from the same laboratory in another material indicates that there must be some undiscovered cause for the wild values.

This example again illustrates the unreliability of estimates derived from single materials and emphasizes the necessity for removal of wild values which contaminate otherwise acceptable data. Furthermore, it is becoming evident that the pooled data from similar studies form a guide for when outlier removal should be initiated and when it may be stopped.

Although additional Cochran outliers are flagged through-out the series, as indicated by the "C" adjacent to the labo-ratory data in Figure 5, it is unnecessary to remove them. The data with only the wild outliers removed are very accept-able. The removal of additional flagged outliers changes both RSD values by only small amounts.

Removal of data from Laboratory 5 as a rank sum outlying laboratory does little to improve RSD_x, but adds the stigma of 17% outliers. The extreme values from this laboratory for 2 materials were flagged as Cochran outliers at the 98% CL. With the other 4 materials, the consistently high averages

from this laboratory were not of sufficient magnitude to flag an outlier at even the 95% CL.

Outliers in Automated Methods

A different set of outlier rejection rules should be developed for use with automated methods for 2 major reasons: First, the ease with which replicates are run provides a multiplicity of within-laboratory data that are not usually available with manual methods. Second, the within-laboratory results from automated methods tend to be very repeatable. These characteristics result in a data pattern consisting of tight major clusters; even minor deviations in range by one laboratory trigger a Cochran outlier test at the 95% CL. Because the deviations of the flagged points are small in an absolute sense, their removal has little influence on the magnitude of RSD_x, the important precision parameter. Although major outliers appear to be less frequent in automated methods than in manual methods, they seem to be more extreme in value when they do appear. This is an unexpected finding, because such aberrant values should be immediately apparent to an operator who should take steps at once to verify the value. In actual practice, usually only single aliquots of test solutions from individual materials are run. It is prudent, therefore, to rerun any material whose results show a marked deviation from those of similar materials. It would also appear to be wise to rerun a certain fraction of all aliquots in non-adjacent positions in order to note and control the appearance of wild values.

Generalizing the discussion of outlier treatment from the individual studies, particularly from the ferrous sulfate and nitroglycerin materials, the following points are suggested when dealing with collaborative studies of automated methods:

(a) *Dixon test applied to the data from a single laboratory on a single material.*—Although the Dixon test for extreme values was developed to apply to small sets of data, such as the 10 values generated for each material by each laboratory, the usual repeatability of automated methods results in numerous "false alarms" by this test. When viewed from the vantage point of the study as a whole, many such flagged values are seen to have little practical significance, i.e., their removal has little effect on the mean or on the RSDs, unless they are also flagged by one of the other statistical tests. The Dixon test should not be used with the data from individual laboratories because it is too sensitive to the vagaries of the data pattern.

(b) *Dixon test of individual values applied to the entire series of values from a single material.*—Although there are theoretical objections to the Dixon test being applied to individual values when replication is present, this test was very successful in flagging those extreme individual values which were so far from the main body of all the data that they could arise only from a major blunder, such as an incorrect recording of an instrument reading or of a weight. The objection to this application involves the assumption that all the nonoutlying values are part of the same normal population, which obviously is not the case. Furthermore, most Dixon tables stop at 25 values, whereas most data sets from automated methods contain at least 60 data points. In practice, the "25" row is used for the critical value when 25 points are exceeded. This results in a test that is more conservative than necessary, i.e., a value that might actually be a Dixon outlier at the 97% CL would be flagged as an outlier at only the 95% level, or would not be flagged at all. With automated methods, there is a considerable degree of overlap in the results from this test with the results from the Cochran test for extreme ranges from laboratories.

All Dixon tests must be supplemented with a visual examination for masked outliers, i.e., 2 extreme values sufficiently close together that they affect the numerator and/or the denominator of the test statistic so that it does not exceed the critical value when it is obvious by inspection or by another test that either value in the absence of the other would be classified as a Dixon outlier. If this masking effect is not caught by inspection, it may be caught by the Cochran or Grubbs tests.

Occasionally, we find empirically that the Dixon test is not triggered when the number of measurements coincides with a transition point in the Dixon tables (at 7, 10, and 13). The Grubbs test is a valuable supplement to handle such cases. In most other cases, the results of the Grubbs test parallel the results of the Dixon test.

(c) *Dixon test applied to laboratory averages.*—This test is most successful in flagging laboratories that have probably incurred a calibration error from the use of incorrect standards, thus causing a shift of all values from that laboratory. But calibration errors are more likely to be operator-generated than method-generated, and their appearance in a collaborative study should cause an immediate investigation by comparison of the standard solution(s) used with a freshly (and presumably more carefully) prepared standard solution. Because automated methods usually have a minimum number of participating laboratories, often only 6, the removal of an entire laboratory flagged by this test (17% of the data) leaves very little room for the removal of any other outlying data. The appearance of a second potential outlying laboratory caused by reiterating this test poses the threat of aborting the entire study because 33% outliers is excessive by any standard.

Outliers flagged by the Dixon test applied to laboratory averages (denoted by "D" and a CL in Table 1) have been much less frequent in studies of automated methods than in those of the other 2 types of methods (chromatographic separation/spectrophotometric measurement and GC) examined in the previous 2 papers of this series.

(d) *Cochran outliers.*—Cochran outliers have been the most frequent type of outliers from automated method studies. One laboratory may present a range of values considerably greater than the ranges of other laboratories. When the average of the laboratory flagged as a Cochran outlier is not the highest or lowest of the group, the removal of that laboratory has little effect on RSD_x, although it could reduce RSD_o substantially. Because it was usually a single value that triggered the outlier signal, it appeared that a Dixon test applied laboratory by laboratory to the 10 replicates from each laboratory individually might give the same result as the Cochran test. This outlier test, designated as the "Dixon internal laboratory outlier test," as discussed previously, proved to be too sensitive for practical application.

Whereas the Dixon tests check the position of each extreme value relative to its neighbor (or second nearest neighbor), the Cochran test checks the range (technically the variance) of the laboratory against all the other ranges in the series. The computer program prints out the fraction of the total variance arising from the extreme range. Therefore, the Cochran test is very good at flagging a laboratory with a wide dispersion of results relative to the other laboratories, but where no single or average value is an extreme point. The Cochran test signals, however, often overlap those of the Dixon test applied to individual values. Cochran test signals can often be ignored when the RSD_x is acceptable even when

the outlier is included, because the test is triggered when the values from most of the laboratories are very "tight," i.e., very close together. A sufficient number of cases exist, however, where only the Cochran test signaled the presence of an outlier having a significant effect on RSD_x to warrant its retention as a primary outlier test.

When a laboratory contributes only 2 or 3 values per material, there is no problem associated with discarding the results from that laboratory for that material, because at least 2 values contributed to triggering the Cochran test response. However, in an automated method study, although 2 values may trigger the test, there are often 8 or 9 intermediate innocent bystander values that seem to be part of the general population. Although removal of all the values for that material from that laboratory would not significantly change the RSDs from those obtained by merely removing the extreme values, the percent outliers is unduly inflated. In this respect, the accusation against the Youden rank sum test as unjustly eliminating values which are part of the normal population, in many cases, also applies to eliminating the intermediate values in a flagged Cochran outlying laboratory. In dealing with automated methods, therefore, a laboratory flagged as a Cochran outlier should have only the extreme value (or the 2 extreme values when the values at both ends appear to be outliers) removed in the next iteration.

(e) *Grubbs test.*—The Grubbs test checks the most extreme deviation from the average against the standard deviation of all the values. It is used as a "back-up" test to cover the known deficiencies in the Dixon tests—masking and discontinuities.

(f) *Youden rank sum test.*—The Youden rank sum test plays only a minor role in examining the results from collaborative studies. Most of the studies contained fewer than the 5 materials necessary for its useful application. In the one case (nitroglycerin) where it was pertinent, its signals proved to be redundant.

Data Summary for Automated Methods

The data from a total of 2461 determinations for 38 dosage forms of 14 drugs from 10 collaborative studies using an average of 7 laboratories per study are given in Table 1 and summarized in Table 2. Automated methods may be roughly characterized by an average RSD_x of 1.9%, increasing very slightly with decreasing analyte concentration, an average RSD_o of 1.1%, a RSD_o/RSD_x ratio of 0.57, and an average outlier rate of 0.57% (of determinations).

Table 3 summarizes the outlier removal that was done to obtain acceptable RSD_x values. Again, despite the fact that every study contained at least one material with outliers flagged by the statistical tests used, data from only 4 of the 38 materials (11%) required that the outliers be removed to obtain acceptable RSD_x values. Three of the 4 indicated outliers were flagged by the Dixon test as applied to individual values at the 99% CL as well as by the Cochran test at the 99% CL. The removal of these single values reduced the RSD_x substantially. In the fourth case (reserpine, 0.1 mg/tablet), 2 different types of outliers were flagged simultaneously. Two individual values from Laboratory 4 were flagged as Cochran outliers at the 99% CL, but their removal hardly affected the RSD_x. The average value from Laboratory 5 was flagged by the Dixon test as applied to laboratory averages but at only the 95% CL. In this case a 14% outlier rate (10 of 70 values) had to be accepted to bring the RSD_x in line with the other values for RSD_x in the same series. As in the previous papers, the Grubbs and the rank sum tests were not needed.

The final RSD_x data are presented in Figure 1. The center line represents the line of best fit for RSD_x vs $-$log concentration (expressed as a decimal fraction) but labeled linearly, bracketed by its 95% prediction limits (the region within which 95% of such future regression lines will fall). The reproducibility RSD_x taken off the line of best fit extends from about 1.6% at the 100% concentration point to 2.2% at the 0.1% concentration point, a change of 0.2% RSD_x per 10-fold concentration change.

As in the previous studies, the RSD_x for automated methods appears to be independent of the nature of the analyte and of the matrix. It should be emphasized that although much of this discussion deals with handling outliers, only about 0.6% of the reported values fall in the rejectable category.

REFERENCES

(1) Horwitz, W., & Albert, R. (1984) *J. Assoc. Off. Anal. Chem.* **67**, 81–90
(2) Horwitz, W., & Albert, R. (1984) *J. Assoc. Off. Anal. Chem.* **67**, 648–652
(3) Margosis, M. (1971) *J. Assoc. Off. Anal. Chem.* **54**, 600–602
(4) Committee on Interlaboratory Collaborative Studies (1984) *J. Assoc. Off. Anal. Chem.* **67**, 432–440
(5) *Official Methods of Analysis* (1984) 14th Ed., AOAC, Arlington, VA
(6) Youden, W. J., & Steiner, E. H. (1975) *Statistical Manual of the AOAC*, AOAC, Arlington, VA
(7) Horwitz, W. (1977) *J. Assoc. Off. Anal. Chem.* **60**, 1355–1363
(8) Horwitz, W. (1934) *CHEMTECH* **14**, 186–191

CHEMICAL CONTAMINANTS MONITORING

The FDA Surveillance Index for Pesticides: Establishing Food Monitoring Priorities Based on Potential Health Risk

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Through an approach called the Surveillance Index (SI), the U.S. Food and Drug Administration (FDA) is systematically evaluating the potential health risk of pesticides that may be present as residues in foods. The SI is being used as a primary tool in the establishment of monitoring priorities. Each pesticide is classified and assigned to one of 5 levels of potential risk. The SI documents that are prepared for individual pesticides (a) summarize the information used to assess potential dietary exposure and health risk, (b) provide the rationale for the assigned classification, and (c) identify needs and actions necessary to accomplish appropriate monitoring of the food supply. Reasons are described for the development of the SI, the content of the SI Documents, and the use of the SI in planning and redirecting FDA pesticide monitoring activities.

In 1978, the Commissioner of Food and Drugs appointed a Study Group on Food and Drug Administration (FDA) Residue Programs to critically examine the agency's monitoring and enforcement programs for residues of pesticides and industrial chemicals in food. The Study Group reported its findings and recommendations in June 1979 (1). One of the major issues addressed was the selection of chemicals for monitoring; the Study Group concluded that monitoring should be based primarily on potential health risk, rather than on the capabilities of FDA's commonly used multiresidue analytical methods. They recommended that FDA develop a Surveillance Index (SI) to reflect the relative potential health risk of pesticide and industrial chemical residues that may occur in food and feed commodities, and use the SI to establish monitoring priorities. A number of parameters relating to production and usage, toxicity, environmental fate, and potential for exposure were to be considered.

The development of the SI was initiated by FDA's Center for Food Safety and Applied Nutrition (CFSAN), formerly the Bureau of Foods, in mid-1979. The SI includes a 5-level classification of potential risk to establish monitoring priorities, and SI Documents for individual pesticides, which (a) summarize the information used to assess dietary exposure and health risk, (b) provide the rationale for the classification assigned, and (c) identify needs and necessary actions to accomplish appropriate FDA monitoring. The SI concept extends beyond its written component, however, to encompass *use* of the information to plan and make appropriate changes in FDA monitoring programs.

Development of the Surveillance Index

Three primary questions were initially addressed:
What classification or ranking system should be used?
What information should be reviewed, and where can it be obtained?
What scheduling should be followed in evaluating and classifying the large number of pesticides to be covered?

Classification

A 5-level risk classification was developed after consideration of several ranking approaches (2–4) as well as a less detailed and formalized classification system used earlier to

guide monitoring plans based on EPA's Rebuttable Presumption Against Registration (RPAR):

Class I: The pesticide represents a high health hazard on a toxicological basis. Because of demonstrated adverse effects in animals and/or humans and anticipated significant dietary exposure, the pesticide warrants immediate inclusion in the monitoring program on a continuous basis.

Class II: A high health hazard has not been demonstrated, but there is evidence of possible high risk toxicity effects combined with the potential for significant human dietary exposure. The potential hazard is sufficient to warrant, as soon as possible, a temporary inclusion of the pesticide in the monitoring program until exposure to the pesticide is more clearly defined, or until additional toxicity data, exposure data, or EPA actions indicate assignment to a different class.

Class III: A moderate hazard profile, based on weighing both toxicity and dietary exposure factors, warrants the pesticide's periodic inclusion in the monitoring program over the long term due to the chances of exceeding tolerances or the Acceptable Daily Intake.

Class IV: Sufficiently low hazard potential, from the toxicological and/or exposure standpoint, justifies only intelligence-related monitoring efforts. ("Intelligence-related monitoring" refers to monitoring based on current area-specific information, such as indications of chemical spills or other accidental contamination incidents, of misuse, or of extraordinarily heavy usage of the pesticide.)

Class V: Very little potential hazard, due to low toxicity or minimal possible exposure, warrants exclusion of the pesticide from routine monitoring efforts at this time.

The use of precise numerical ranking, scoring formulas, etc., was avoided because these approaches tend to indicate a degree of precision (and absence of subjectivity) which often is not justified by the accuracy and completeness of the supporting information or by the assumptions used. Precise numerical ranking often leads to inflexible application; an effective and dynamic monitoring program must include the flexibility to respond to localized problems, to quickly utilize new information (changing usage, additional toxicological findings), and to make the most efficient use of available resources. The 5-level SI classification allows the needed flexibility while providing sufficient assessment of potential risk to set priorities for monitoring and for analytical method development.

Surveillance Index Documents

For each pesticide, those parameters that influence the likelihood of dietary exposure and the toxicological information that indicates the potential risk are summarized in a Surveillance Index Document (SID). This information furnishes the basis for placing the pesticide in one of the above 5 classifications. The SID also provides a valuable reference to personnel involved in monitoring and analytical activities.

The primary, and in some cases the only, source of this information is that which is submitted to the U.S. Environ-

Table 1.   Surveillance Index document outline

Pesticide name	Type	SI Class
CFR Reference(s):		
Chemical Name(s):		
Trade Name(s):		
Common Name(s):		
Chemical Structure		Empirical Formula
Molecular Weight:		
Physical State:		
Melting Point:		

I. Exposure and Use Factors
  A. Production/Usage Volume
  B. Chemistry
    1. Solubilities
    2. Stability
    3. Manufacturing Process/Impurities
    4. Degradation
    5. Environmental Stability
      a. Foliar Degradation/Plant Metabolism
      b. Aqueous Degradation
      c. Soil Degradation
      d. Animal Metabolism
    6. Bioaccumulation Potential
    7. Soil Mobility
    8. Systemic Plant Activity
    9. Significant Terminal Residues
    10. Related Pesticides/Common Metabolites
  C. Uses and Potential Exposure
    1. Registered Uses
    2. Tolerances
    3. Known Foreign Uses
    4. Exposure via Foods
    5. Other Exposure Routes
II. Toxicity
  A. Acute Oral LD_{50} (rats)
  B. Oncogenicity
  C. Mutagenicity
  D. Teratogenicity/Reproductive Effects
  E. Other Acute and Chronic Effects
  F. ADI (EPA)
  G. ADI (FAO/WHO)
  H. Acute and Chronic Effects Triggering RPAR (if applicable)
  I. RPAR Status
III. Overall Appraisal
IV. Surveillance Index Classification with Justification
V. Necessary Action

mental Protection Agency (EPA) in support of pesticide registrations and tolerances. The Office of Pesticide Programs, EPA, has contributed extensively to the development of the SI by furnishing summaries or evaluations of these data to FDA.

Scheduling.—U.S. tolerances for residues on foods have been established for more than 300 pesticides; many additional pesticides have the potential for contaminating foods because of significant use in foreign countries or because they are environmental contaminants. The task of evaluating and compiling SIDs for this great number of chemicals was expected to require several years; thus, broad priorities had to be established to determine the general order in which the pesticides would be covered. Based on experience and readily available data, CFSAN scientists placed pesticides that have U.S. tolerances in several groups. Pesticides for which EPA has issued an RPAR were of first priority for SID preparation, whereas those routinely determined by FDA multiresidue analytical methods were of lowest priority; first-hand knowledge and other readily available information on past monitoring, volume and type of use, chemical and physical properties, and toxicological concerns were weighed in scheduling the remaining pesticides for evaluation.

Surveillance Index Document content.—Table 1 shows the outline of the topics addressed in the SID. The classification of each pesticide is assigned by weighing both the toxicological risks and the likelihood and magnitude of dietary exposure. The SID also identifies needs and actions that must be addressed to accomplish appropriate monitoring for the pesticide.

Exposure factors include the manufacturing process scheme; volume, pattern, and type of use; rates of degradation; environmental fate; potential for transfer of residues to meat, milk, eggs, and fish; and information on impurities and alteration products which may also be of toxicological concern. These factors, combined with data from FDA monitoring programs, Total Diet Studies, or worst-case estimates, based on tolerance level residues on all commodities for which the pesticide is registered, provide the basis for assessing potential dietary exposure.

The toxicity section briefly summarizes the toxicological data base; it includes conclusions reached from studies of acute and chronic toxicity, oncogenicity, mutagenicity, teratogenicity, reproductive effects, neurotoxicity, potentiation by other pesticides, and tests on metabolites and impurities. Acceptable Daily Intakes (ADIs) established by EPA and the Food and Agriculture Organization of the United Nations/World Health Organization (FAO/WHO) are also included.

Because much of the information received from EPA is based on confidential information from pesticide manufacturers, the SID often uses secondary references reflecting the overall summary conclusions of responsible scientists rather than providing sufficiently detailed data to permit independent reevaluation. The information presented does not reflect exhaustive literature or information searches, but sources other than EPA are often utilized. These include evaluations of the Joint Meeting on Pesticide Residues of the FAO/WHO and information from the National Cancer Institute, U.S. Department of Agriculture, National Institute of Occupational Safety and Health, the literature, and the considerable background information available within FDA.

Establishment of Monitoring Priorities

After review of the information by CFSAN chemists and toxicologists, the pesticide is classified and necessary actions are recommended. The recommendations may include changes in monitoring frequency, initiation of special surveys, investigations of analytical methodology, selection of appropriate locations and commodities for sampling, and identification of commodities that are major contributors to possible dietary exposure.

Distribution of SI Documents

To date, more than 140 pesticides have been evaluated and classified. The SIDs have been distributed to FDA headquarters, field personnel, and other state and federal agencies; they are also available to the general public from the National Technical Information Service (5). Current projections call for the evaluation of 30–50 additional pesticides per year.

Review and Update of SI Documents

When appropriate, the SID may be revised, and the pesticide may be reclassified. Factors that may warrant reclassification include RPAR decisions, studies demonstrating previously unrecognized toxicological effects, invalidations of past studies, revisions of the Acceptable Daily Intake, and changes in usage.

Use of the Surveillance Index

The SI is used to redirect FDA monitoring programs as individual pesticides are evaluated. Special surveys have been initiated for a number of Class I, II, and III pesticides for which FDA monitoring was limited. Some of these com-

pounds were in the RPAR process; data generated by these surveys have enabled EPA to better estimate dietary exposure and have assisted that agency in reaching RPAR and other regulatory decisions.

The SI is an integral part of the planning of the pesticide portion of the FDA Chemical Contaminants program. In addition to identifying pesticides and commodities that deserve changes in monitoring emphasis, the SIDs aid FDA Districts in designing intelligence gathering activities and sample collections.

The SI is also used in deciding priorities for the development of analytical methods for individual pesticides or groups of pesticides, in identifying compounds that need testing through existing multiresidue analytical methods commonly used by FDA, and in evaluating those past FDA monitoring results that were obtained by using analytical methods that may not have measured the toxicologically important residues of a particular pesticide(s).

The SI is also a valuable reference that enables agency and other government personnel to quickly respond to inquiries regarding the properties, regulations, or past monitoring of specific pesticides.

Summary

The FDA Surveillance Index provides evaluations of the potential health risk of pesticides via dietary exposure. Individual pesticides are assigned to one of 5 risk classes that indicate priorities for monitoring. Gaps in monitoring and analytical methodology are identified, and recommendations are made for filling the gaps and redirecting sampling efforts.

The SI will be expanded to cover additional pesticides, will be updated as necessary and will continue to play a key role in the planning and redirection of FDA monitoring activities.

REFERENCES

(1) Study Group on FDA Residue Programs (1979) *FDA Monitoring Programs for Pesticide and Industrial Chemical Residues in Food.* HEW Publication No. (FDA) 79-2116, U.S Department of Health, Education and Welfare, Rockville, MD
(2) Oller, W. L., Cairns, T., Bowman, M. C., & Fishbein, L. (1980) *Arch. Environ. Contam. Toxicol.* **9**, 483–490
(3) Von Rumker, R., Lawless, E. W., & Meiners, A. F. (1975) *Production, Distribution, Use and Environmental Impact Potential of Selected Pesticides,* U.S. Environmental Protection Agency, Washington, DC. EPA 540/1-74-001, pp. 347–356
(4) Gevertz, J. N., Bild, E., & Sellers, D. W. (1980) *Chemical Selection Methods: An Annotated Bibliography,* U.S. Environmental Protection Agency, Washington, DC, EPA 560/TIIS-80-001
(5) *The Surveillance Index* (August 1981) Publications Section, National Technical Information Service, 5285 Port Royal Rd, Springfield, VA 22161. NTIS Accession No. PB 82-913200 covers a continuing subscription to the Surveillance Index to include updates.

Disappearance of Organochlorine Residues from Abdominal and Egg Fats of Chickens, Ontario, Canada, 1969–1982

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Abdominal fats were collected from 8-week old broilers slaughtered at provincially inspected abattoirs across Ontario between 1969 and 1982. Domestically produced hen eggs were collected from either egg grading stations or producers over the same period. Composite samples were analyzed for organochlorine insecticides and industrial chemicals. Between 1979 and 1982, organophosphorus insecticides were routinely included in these analyses. ΣDDT and PCB residues declined rapidly between 1969 and 1982 in extractable lipids of both abdominal and egg fats, while dieldrin declined less markedly over the same period. Declines in residues followed first order logarithmic regression equations. Chlordane and heptachlor epoxide were rarely observed above the detection limit of 1 μg/kg in egg fat; however, the incidence of these residues in abdominal fat increased after 1973 following the removal of aldrin, dieldrin, and heptachlor in 1969 and the subsequent increased use of chlordane for soil insect control until 1977. Lindane residues were rarely observed above the detection limit. In 1979, when the detection limit was reduced, both α-BHC and lindane were identified, but at levels below 1 μg/kg. Endosulfan, methoxychlor, and fenthion were identified on one or 2 occasions over the 13-year period.

Pesticide residues in chicken body and egg fats can be derived from either topical treatment of birds, spraying of poultry housing, or absorption of contaminants from the diet, water,

or litter. Persistent organochlorine insecticides, while not used directly on poultry, do nevertheless appear in tissues indirectly from use of these compounds in agriculture. Industrial organochlorines also appear in tissues from the environmental contamination of feed and water.

The use of organochlorine insecticides in Ontario agriculture has changed markedly over the last decade (1). The persistent insecticides, including DDT, TDE, aldrin, dieldrin, endrin, heptachlor, and toxaphene, were removed from use between 1969 and 1972, while the less persistent insecticides, chlordane, endosulfan, and methoxychlor, were removed from major use by the mid to late 1970s. In 1977, chlordane was removed from the list of insecticides allowed for controlling soil insects in corn, and endosulfan was removed from use in controlling foliar caterpillars on tobacco. During this same period and extending to the present, external parasites were controlled on poultry by the use of carbaryl, coumaphos, and malathion (2). More recently, permethrin has been added to the list of recommended materials.

This survey was initiated in 1969 to monitor the fats of carcasses and eggs for organochlorine insecticides. PCB (polychlorinated biphenyl) analysis was added in late 1969 at a time when the general use of this industrial fire retardant was unrestricted. In 1971, PCBs were voluntarily removed

Table 1. Average recoveries of organochlorine and organophosphorus insecticides, HCB, and PCBs and maximum residue levels (MRL) in extractable fat of chicken eggs and abdominal fat

Compound	Fort. range, μg/kg	Av. rec., % Abdominal fat	Eggs	MRL,[a] mg/kg Abdominal fat	Egg fat
p,p'-DDE	10–100	97	94		
p,p'-DDT	20–200	91	90	1.0	0.5
p,p'-TDE	20–200	89	89		
Dieldrin[b]	10–100	93	90	0.2	<0.1
Heptachlor epoxide[c]	10–100	90	89	0.2	<0.1
Lindane	10–100	94	92	2.0	<0.1
α-BHC	10–100	87	89	0.1	<0.1
α-Chlordane	10–100	93	90	0.1	<0.1
β-Chlordane	10–100	94	89	0.1	<0.1
Endosulfan-1	10–100	89	91		
Endosulfan-2	20–200	90	88	0.1	<0.1
Endosulfan sulfate	50–500	88	84		
HCB	10–100	78	71	<0.1	<0.1
PCBs	100–1000	87	86	<0.1	<0.1
Trichlorfon	200–2000	78	74	<0.1	<0.1
Fenthion	200–2000	96	93	<0.1	<0.1
Malathion	200–2000	89	92	<0.1	<0.1

[a] Others included dicofol, endrin, and methoxychlor = MRL <0.1; tetrachlorvinphos = MRL 0.76 mg/kg in abdominal fat, <0.1 mg/kg in egg fat.
[b] Includes aldrin.
[c] Includes heptachlor.

Table 2. Organochlorine residues in chicken fat and eggs collected across Ontario between 1969 and 1982

Years	No. Composite samples (eggs or carcasses)	Statistic	ΣDDT Antilog	Log	Dieldrin Antilog	Log	Heptachlor epoxide	Chlordane	Lindane	PCB Antilog	Log
Abdominal Fat:											
1969–70	19 (95)	Mean	391	2.592	28	1.443	<1	NM[a]	9	946	2.976
		SD		0.266		0.526	—		18		0.305
1971–72	21 (81)	Mean	114	2.058	7.7	0.889	<1	NM	2	54	1.736
		SD		0.527		0.611	—		11		0.467
1973–74[b]	17 (49)	Mean	35	1.541	6.3	0.799	14	NM	<1	195	2.289
		SD		0.589		0.674	44		—		0.418
1975–76	9 (30)	Mean	139	2.142	20	1.3C6	6	22	<1	391	2.592
		SD		0.467		0.447	18	20			0.452
1979–80	21 (102)	Mean	7.6	0.880	1.5	0.170	1.1	1.0	0.7[c]	6.5	0.814
		SD		0.476		0.416	0.2	1.0	2.0		0.159
1981–82[b]	50 (180)	Mean	2.3	0.369	0.74	0.132	4.6	4.6	0.4	4.8	0.681
		SD		0.390		0.230	2.5	10.0	0.6		0.297
Total	137 (537)										
Eggs:											
1969–70[d]	30 (120)	Mean	188	2.974	8.3	0.920	<1	NM	5	405	2.607
		SD		0.759		0.556	—		20		0.106
1971–72	31 (124)	Mean	177	2.247	4.9	0.693	<1	NM	<1	209	2.321
		SD		0.625		0.438	—		—		0.416
1973–74	22 (64)	Mean	137	2.138	10.5	1.020	<1	NM	<1	255	2.406
		SD		0.322		0.221	—		—		0.340
1978–80	4 (10)	Mean	27	1.435	1.3	0.122	1.5	<1	2.4	126	2.100
		SD		0.416		0.488	1.0		2.0		0.328
1981–82	31 (101)	Mean	3.9	0.465	<0.5		<1	<1	<1	<10	—
		SD		0.490		—	—	—	—	—	—
Total	118 (419)										

[a] NM = not measured.
[b] Endosulfan and its metabolite found in 6% of abdominal fat samples at 160 μg/kg (1973–74) and in 2% of samples at 5.4 μg/kg (1981–82).
[c] α-BHC identified in addition to lindane at 0.2 ± 0.1 μg/kg in 1979–80 and 0.6 ± 0.8 μg/kg in 1981–82.
[d] Methoxychlor found in 3% of eggs in 1969 at a mean of 390 μg/kg in the extractable fat.

from sale into open system use, and in 1976, PCBs were regulated under the Environment Contaminants Acts and restricted to use in electrical transformers and capacitors.

Monitoring for organophosphorus insecticides was done on a few samples in 1972–74; however, it was not until 1979 that this analysis was added to the routine analysis procedure.

Methods and Materials

Collection of Samples

Composite samples of abdominal fat were collected from 3–5 carcasses of 8-week old broilers by members of the Veterinary Services Branch, Ontario Ministry of Agriculture and Food, shortly following slaughter at provincially inspected abattoirs. Between 1969 and 1976, samples were also collected by officials of the Ontario Ministry of the Environment. Samples of abdominal fat were collected at random from carcasses on an assembly line, as were egg samples at the farm level or at a grading station. Egg samples came from flocks that varied in age between 15 and 36 weeks old. Samples were collected from all regions of the province; in the case of abdominal fats, 30% came from the southern, 25% from the eastern, 24% from the western, 16% from the central, and 5% from the northern region; in the case of eggs, 32% came from the southern, 27% from the eastern, 25% from the western, 10% from the central, and 6% from the northern region. The sites were not always the same because of changes in private sector businesses. Field staff

Table 3.   Distribution of ΣDDT and PCBs in composite samples of chicken fat and eggs collected in Ontario between 1969 and 1982

Compound	Tissue	Period	Frequency distribution (%) of residues (μg/kg)				DDE (% of ΣDDT)
			<10	11–100	101–1000	1000 +	
ΣDDT	Fat	1969–70	0	0	95	5	57
		1971–72	0	57	38	5	54
		1973–74	12	75	13	0	79
		1975–76	0	22	78	0	71
		1979–80	95	5	0	0	95
		1981–82	94	6	0	0	63
	Eggs	1969–70	3	37	37	23	70
		1971–72	0	45	45	10	85
		1973–74	9	35	52	4	84
		1978–80	50	50	0	0	83
		1981–82	84	16	0	0	94
PCB	Fat	1969–70	0	70	10	20	
		1971–72	5	29	62	4	
		1973–74	25	50	25	0	
		1975–76	0	36	55	9	
		1979–80	95	5	0	0	
		1981–82	84	16	0	0	
	Eggs	1969–70	0	20	80	0	
		1971–72	3	48	45	4	
		1973–74	0	13	87	0	
		1978–80	10	65	25	0	
		1981–82	50	50	0	0	

Table 4.   Distribution of cyclodienes in composite samples of chicken fat and eggs collected in Ontario between 1969 and 1982

Cyclodiene	Tissue	Period	Frequency distribution (%) of residues (μg/kg)			
			<1	1–10	11–100	101–1000
Dieldrin	fat	1969–70	5	16	68	11[a]
		1971–72	14	57	24	5[a]
		1973–74	25	51	24	0
		1975–76	0	33	67	0
		1979–80	43	57	0	0
		1981–82	76	24	0	0
	eggs	1969–70	7	60	30	3
		1971–72	19	71	10	0
		1973–74	14	59	27	0
		1978–80	50	50	0	0
		1981–82	100	0	0	0
Heptachlor epoxide	fat	1969–70	0	95	5	0
		1971–72	0	100	0	0
		1973–74	0	100	0	0
		1975–76	0	89	11	0
		1979–80	40	60	0	0
		1981–82	50	46	4	0
	eggs	1969–70	97	3	0	0
		1971–72	100	0	0	0
		1973–74	100	0	0	0
		1978–80	75	25	0	0
		1981–82	100	0	0	0
Lindane	fat	1969–70	70	20	10	0
		1971–72	95	0	5	0
		1973–74	95	5	0	0
		1975–76	100	0	0	0
		1979–80	90	10	0	0
		1981–82	100	0	0	0
	eggs	1969–70	82	0	18	0
		1971–72	95	5	0	0
		1973–74	100	0	0	0
		1978–80	75	25	0	0
		1981–82	100	0	0	0

[a] Two composite samples exceeded the 200 μg/kg MRL, one 700 μg/kg in 1970 and one 400 μg/kg in 1971.

collected samples throughout the summer on the basis of work priorities and hence, in some years, no samples could be obtained. The size of the composite was intended to bear some relation to the number of birds slaughtered or the number of eggs involved.

Analytical Procedures

Where only organochlorine residues were determined, poultry fat samples were fluidized by warming in an oven at 100°C; eggs were mixed thoroughly with anhydrous sodium sulfate and exhaustively extracted with hexane in a Soxhlet apparatus, followed by removal of the solvent by rotary evaporation. Subsamples of fluidized fat (1.0 g) were then cleaned up according to the procedure of Frank et al. (3). PCBs were fractioned from organochlorine insecticide according to the procedure of Armour and Burke (4) and later (after 1972) according to the procedures of Berg et al. (5) and Holdrinet (6). Where organophosphorus insecticides were also determined, extractions were carried out by blending with acetonitrile–water (2 + 1) according to the procedure of Braun and Lobb (7), a measured portion of the extract was cleaned up for organochlorine analysis according to the method of

Table 5. HCB and organophosphorus residues (OP) in abdominal fat and egg fat collected across Ontario between 1972 and 1982

Contaminant	Year	Tissue	No. of composite samples	No. of eggs or carcasses	Content in extractable fat, µg/kg	
					Mean	SD
HCB	1972–74	abdom. fat	5	21	9	20
		egg fat	24	84	108	120
	1981–82	egg fat	31	96	0.6	2.0
OP	1973–74	abdom. fat	3	12	ND[a]	—
	1979–80	abdom. fat	21	100	54[b]	211
		egg fat	4	10	ND	—
	1981–82	abdom. fat	50	100	ND	—
		egg fat	31	96	ND	—

[a] Less than detection limits of 10 µg/kg for fenthion and malathion, 50 µg/kg for tetrachlorvinphos.
[b] Fenthion found in 9.5% of samples; in one sample, the residue exceeded the MRL of 100 µg/kg (i.e., 950 µg/kg).

Table 6. Organochlorine residue disappearance rates[a]

Tissues	Contaminant	Equation Log$_{10}$y =	F-Test	r^2	Half residue disappearance, years
Abdominal fat	ΣDDT	2.546 − 0.168x	22.8	0.85	1.8
	dieldrin	1.402 − 0.116x	12.0	0.74	2.6
	PCB	293 − 214x	9.4	0.70	1.8
Egg Fat	ΣDDT	2.48 − 0.132x	23.0	0.88	2.3
	dieldrin	1.05 − 0.100x	15.4	0.84	3.0
	PCB	2.67 − 0.104x	7.9	0.72	2.9

[a] y = residue in µg/kg, and x = time in years.

Mills et al. (8), and the remainder of the extract was concentrated and analyzed directly using phosphorus-specific detection. Before 1976, organochlorines were determined by gas chromatography using 1.8 m × 2 mm id columns packed with 4% SE-30/6% QF-1 on 80–100 mesh Chromosorb W and electron capture detection (^{3}H and ^{63}Ni) operated in the constant dc polarizing mode. From 1976 to 1980, organochlorine determinations were made on 1.8 m × 2 mm id columns packed with 1.5% OV-17/2.0% OV-210 on 100–120 mesh Gas-Chrom Q and with ^{63}Ni electron capture detectors operated in the constant current mode. After 1980, organochlorine insecticides were determined by capillary column gas chromatography (fused silica, 15 m × 0.25 mm, 0.25 µm coating of SE-54) and constant current electron capture detection, while PCBs were determined by packed column as in previous years.

Organophosphorus insecticides were determined by gas chromatography on 1.8 m × 2 mm id columns packed with 5% OV-1 on 100–120 mesh Gas-Chrom Q with flame photometric detection (P mode, 525 nm filter) and using temperature programming from 150 to 240°C at 5°C/min. Recoveries were determined in each sampling period by fortification of fluid fat and pureed egg just before extraction and then carrying through the procedure as described. Table 1 lists averaged recoveries; these data were not corrected for recoveries.

Detection limits were about 1 µg/kg in egg fat for organochlorine insecticides and 10 µg/kg for PCBs, while those for organophosphorus residues varied from 10 µg/kg (dichlorvos, trichlorfon, chlorpyrifos, fenchlorphos, fenthion, crufomate) to 200 µg/kg (tetrachlorvinphos, phosmet, coumaphos). PCBs were identified and quantitated by comparison with mixtures of Arochlors 1254 and 1260 and checking for resemblance to peaks VII, VIII, and X according to the system of Reynolds (9).

Statistics

Because of outliers in the data, the results for ΣDDT, dieldrin, and PCB were transformed into logarithms for statistical and regression analyses.

Results

Abdominal Fat

Between 1969 and 1982, a total of 137 composite samples were analyzed, representing 537 carcasses (Table 2). ΣDDT residues declined exponentially over the 13-year period from a high mean residue of 391 µg/kg in 1969–70 to 2.3 µg/kg in 1981–82. This is further illustrated by the frequency distribution of sample residues in Table 3. The percentage of fat samples with less than 10 µg/kg increased from zero in 1969–70 to 94% in 1981–82. Only between 1969 and 1972 did residues exceed the maximum residue limit (MRL) of 1 mg/kg, and during this period, 5% of samples exceeded this level (Tables 1 and 3).

Residues of dieldrin declined from a mean of 28 µg/kg in 1969–70 to a mean of 0.74 µg/kg in 1981–82 (Table 2). This change in residue level can be observed in the frequency distribution where 79% of samples exceeded 10 µg/kg in 1969–70, and none exceeded the level in 1981–82 (Table 4). Dieldrin residues that exceeded the 200 µg/kg MRL were found in only 2 composite samples, one occurring in 1970 (700 µg/kg) and one in 1971 (400 µg/kg) (Tables 1 and 4).

Heptachlor epoxide appeared in tissues in 1973–74 following the cancellation of agricultural uses of aldrin and dieldrin. Chlordane, which contained 11% heptachlor, was applied to soil as a replacement compound for controlling insects in corn and other crops. The frequency distribution for heptachlor epoxide reveals that an increase in residue occurred in 1975–76, but then decreased by 1981–82 (Table 4). This appeared to coincide with the rescinding of the major uses for chlordane in late 1977. At no time was the MRL exceeded for either heptachlor epoxide (200 µg/kg) or chlordane (100 µg/kg) (Table 1).

Lindane residues declined from 9 µg/kg in 1969–70 to 0.4 µg/kg in 1981–82. At no time was the 2 mg/kg MRL exceeded (Tables 1, 2, and 4). Between 1979 and 1982, trace amounts of α-BHC were detected along with lindane and were of a similar order of magnitude (Table 2). Endosulfan and its sulfate metabolite were detected in 2 composite samples (Table 2).
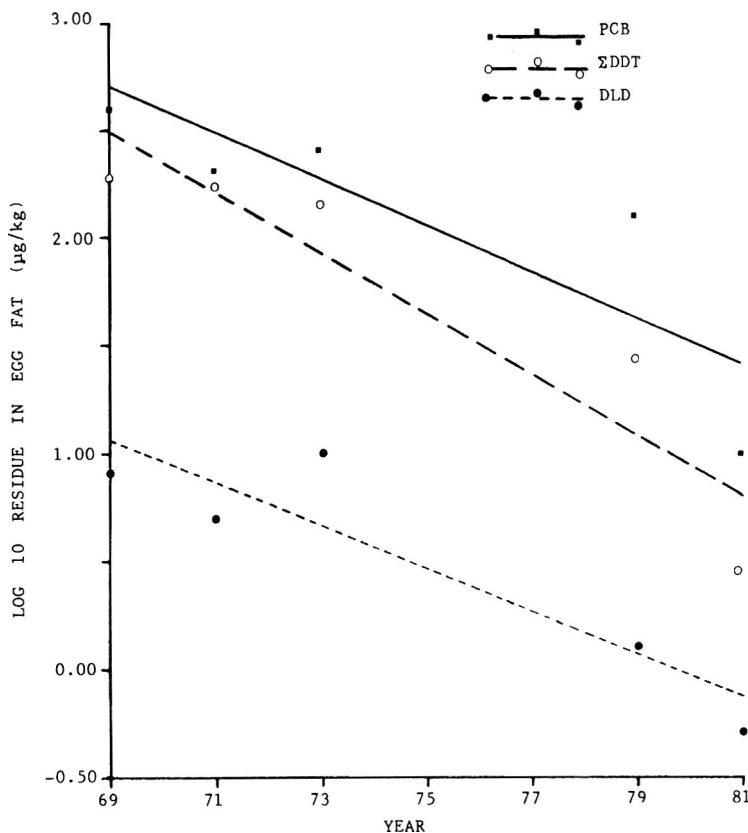
Figure 1.   Decline In ΣDDT, dieldrin, and PCB in egg fat (hens) between 1969 and 1981, Ontario, Canada.

Polychlorinated biphenyl (PCB) residues declined from a mean of 946 μg/kg in 1969–70 to 4.8 μg/kg in 1981–82 and reflected the action taken against the uses of PCBs mentioned above (Table 2). No MRL was established for avian fat, hence a negligible residue of 100 μg/kg is assumed as an actionable level. The frequency distribution reveals that in 1969–70, 30% of samples had levels exceeding 100 μg/kg MRL, but by 1981–82 all sample residues were below this level (Table 3).

Analysis of hexachlorobenzene (HCB) was performed on a few composite samples between 1972 and 1974. At that time, samples were found to contain mean residues of only 9 μg/kg (Table 5).

A few samples were analyzed for organophosphorus insecticides in the 1973–74 period, and no residues were found (Table 5). Between 1979 and 1982, analysis for organophosphorus compounds became routine. In 1979–80, fenthion was identified in 9.5% of samples, and in one sample, the residue exceeded the MRL of 100 μg/kg (i.e., 950 μg/kg) (Table 5).

No residues of endrin, mirex, or toxaphene were detected throughout the 13-year period. A first order logarithmic regression equation described the disappearance of ΣDDT, dieldrin, and PCB from successive collections of abdominal fat over the 13-year period (Table 6). The r^2 values and the half residue disappearance were similar for all 3 compounds.

Egg Fat

Between 1969 and 1982, a total of 118 composite samples were analyzed representing 419 eggs (Table 2). ΣDDT residues declined markedly over the 13-year period from a high mean level of 188 μg/kg in 1969–70 to a low mean level of 3.9 μg/kg in 1981–82. The frequency distribution shows that in 1969–70, 60% of composite egg samples were above the negligible residue level of 100 μg/kg assumed under the Food and Drug Act (1982) (Tables 1 and 3). By 1981–82, only 16% of

composite samples were identified that exceeded 10 μg/kg and none exceeded 100 μg/kg.

Residues of dieldrin declined from a mean level of 8.3 μg/kg in 1969–70 to less than the detection level of <0.5 μg/kg in 1981–82 (Table 2). Three percent of eggs in the 1969–70 period exceeded the 100 μg/kg negligible residue level; however, by 1981–82 all composite egg samples contained residues <1 μg/kg (Tables 1 and 4).

Heptachlor epoxide and chlordane were rarely detected in eggs above the 1 μg/kg level (Tables 2 and 4). Lindane appeared in egg fat in the 1969–70 period and again at very low levels in the 1978–80 period; otherwise, no residues were found above the 1 μg/kg detection limit (Tables 2 and 4).

PCB residues declined in egg samples from 405 μg/kg in 1969–70 to <10 μg/kg in 1981–82 (Table 2). These changes reflect the action taken on the use of PCBs outlined above. Because no MRL has been established for PCB residues in eggs, the negligible residue of 100 μg/kg is assumed as a maximum residue limit in egg fat.

Discussion

The levels of terminal pesticide residues in Canadian meat was last reported by Saschenbrecker (10) for the period 1970–74. During that period, mean residues in poultry fat from Ontario were as follows: ΣDDT 48.5 μg/kg, lindane and BHC 0.24 μg/kg, chlordane 0.25 μg/kg, and PCBs 6.3 μg/kg. Methoxychlor, aldrin, dieldrin, heptachlor, and its epoxide were not detected. The means, except for ΣDDT and PCB, were in general an order of magnitude lower than those reported in this paper. For other organochlorines, close agreement was observed.

The rates of disappearance of ΣDDT, dieldrin, and PCB were calculated for regression, and the equations appear in Table 6 and Figure 1 for egg fat. It was evident that the half

residue disappearance from abdominal fat was more rapid than its disappearance from eggs. This might be explained by the shorter life turnover of broilers (8 weeks) as opposed to longer life span of laying hens (14–36 weeks), allowing for greater accumulation over the life of a laying bird and, thus, a greater transfer to the egg. This is supported by the fact that abdominal fat levels were much higher than in egg fat, further illustrating the redistribution from body to egg. PCB residues changed from 30%, which is above the negligible residue level in 1969–70, to none exceeding this level in 1981–82.

Eggs analyzed between 1972 and 1974 contained mean HCB residues of 108 µg/kg; however, by 1981–82, this level had declined to 0.6 µg/kg. No residues or organophosphorus insecticides, endrin, endosulfan, methoxychlor (except 1969), mirex, of toxaphene were identified in eggs over the 13 years of the monitoring survey.

REFERENCES

(1) Frank, R., Braun, H. E., & Fleming, G. (1983) J. Food Prot. 46, 893–900
(2) Ontario Livestock Pesticide Committee (1971, 1972, 1973, 1975, 1976, 1978) "Control of external parasite on poultry," Ontario Ministry of Agriculture and Food, Ontario, Canada, Agdex 450/653
(3) Frank, R., Braun, H. E., & McWade, J. W. (1970) Pestic. Monit. J. 4, 31–41
(4) Armour, J. A., & Burke, J. A. (1976) J. Assoc. Off. Anal. Chem. 53, 761–768
(5) Berg, O. W., Diosady, P. L., & Rees, G. A. V. (1972) Bull. Environ. Contam. Toxicol. 7, 338–347
(6) Holdrinet, M. (1974) J. Assoc. Off. Anal. Chem. 57, 580–584
(7) Braun, H. E., & Lobb, B. T. (1976) Can. J. Anim. Sci. 56, 373–376
(8) Mills, P. A., Bong, B. A., Kamps, L. R., & Burke, J. A. (1972) J. Assoc. Off. Anal. Chem. 55, 39–43
(9) Reynolds, L. M. (1971) Residue Rev. 34, 27–57
(10) Saschenbrecker, P. W. (1976) Can. Vet. J. 17, 158–163

Long-Term Preservation of Egg and Tissue Homogenates for Determination of Organochlorine Compounds: Freezing Versus Freeze-Drying

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Storage of wet egg homogenates at temperatures from −18° to −28°C was more suitable for long-term preservation than freeze-drying. Changes in residue levels of heptachlor epoxide, oxychlordane, dieldrin, hexachlorobenzene, p,p'-DDE, mirex, and PCBs were not significant over a 3-year period in fresh herring gull egg homogenates stored at −18° to −28°C. Compounds with gas chromatographic retention times shorter than hexachlorobenzene vaporized during freeze-drying at a rate proportional to their volatility. Evaporative losses of components with vapor pressures less than hexachlorobenzene did not occur in naturally contaminated herring gull eggs after storage at room temperature for up to 1 year. Higher losses of all compounds, up to 25% for p,p'-DDE, occurred in freeze-dried whole-body herring gull homogenates. Easily dehydrochlorinated compounds were rapidly degraded in freeze-dried chicken egg homogenate at room temperature: The half-life of p,p'-DDT and p,p'-DDD was about 20 days, and that of α- and γ-hexachlorocyclohexane was $\ll$16 days. About one-third of oxychlordane in herring gull eggs was lost in 1 year under these conditions, but none was lost after freeze-drying when the homogenate was stored at −18° to −28°C.

Archiving of tissues and environmental samples for subsequent determination of heavy metals or organic compounds has received considerable attention in the last few years, as indicated by recent conferences on the subject (1, 2). Few definitive answers exist to the problems of long-term preservation for the simple reason that long-term experiments must be performed, and few have been done (3, 4). The problems can be divided into 2 broad areas: preservation of the integrity of the sample (from bacterial, fungal, or enzymatic decomposition and dehydration) and preservation of the levels and chemical integrity of the compounds to be determined. The most serious problems for metals are external contamination of the sample and losses to container surfaces. Decomposition during storage is potentially more serious for organic compounds.

The Canadian Wildlife Service has archived samples of wildlife tissues since the late 1960s under a variety of conditions. Many of the early samples were stored in domestic chest freezers after determination of organochlorine compounds, such as DDT, DDE, and dieldrin, known to be present at that time. PCBs (polychlorinated biphenyls), if determined at all, could be estimated only as Aroclor mixtures because of the lack of sophisticated analytical techniques. In 1973, systematic collection of herring gull eggs from all of the Great Lakes on an annual basis was initiated to determine organochlorine compound trends. More recently, a formal tissue bank has been formed from frozen specimens (2). The egg homogenates were stored in scintillation vials as 6–10 g subsamples: glass vials with aluminum foil-lined caps for organochlorine compound determination, and linear polyethylene vials and caps for heavy metal determination. One subsample was analyzed immediately, and the remainder were archived at −18° to −28°C. The size of the collection soon became large enough that alternative methods of preservation to freezing were considered. In the last few years, the archived samples have also increasingly been used for retrospective determination of trends in levels of newly discovered or newly important residues, such as chlorinated dioxins and chlorinated styrenes. To meet these requirements, studies were needed on long-term preservation of residues in eggs and tissues.

Initially, a project was begun to study the effects of freeze-drying and subsequent storage conditions on residue levels in egg substrates. Because not all compounds of interest were found as natural contaminants, one experiment used artificially contaminated chicken eggs to test the effect of freeze-drying and short-term storage (up to 50 days) on a wide variety of compounds. As a complementary experiment, a pool of naturally contaminated herring gull eggs from the Great Lakes was freeze-dried and stored at 23°C and at −18° to −28°C in the dark for 1 year, and then analyzed at 3-month intervals. This same pool was used over a 3-year period for blind reference samples in the quality assurance programs for chemical analysis of herring gull eggs. Data from this

Table 1. Effect of freeze-drying and short-term storage at room temperature on organochlorine residue levels in homogenized chicken egg artificially contaminated at different concentrations[a]

Compound	Low-level contamination[b]					High-level contamination[c]			
	Nominal spiking level (mg/kg)	% rec., wet sample	% rec., dried, not stored[d]	% rec., dried, stored[e]	Storage time (days)	Nominal spiking level (mg/kg)	% rec., dried, not stored[d]	% rec., dried, stored[e]	Storage time (days)
1,2,3-Trichlorobenzene	0.070	69 (±1.1)	10 (±3.3)	19 (±4.3)	16	0.70	19 / 21	27 / 27	23
1,2,3,5-Tetrachlorobenzene	0.050	82 (±1.1)	15 (±1.1)	26 (±1.1)	16	0.50	38 / 41	36 / 34	23
Pentachlorobenzene	0.010	90 (±1.9)	26 (±2.7)	65 (±1.6)	16	0.10	64 / 69	61 / 61	23
Hexachlorobenzene	0.028	93 (±1.9)	56 (±1.3)	83 (±3.1)	16	0.28	82 / 88	83 / 84	23
α-Hexachlorocyclohexane	0.047	96 (±5.5)	32 (±3.9)	<0.1	16	0.47	43 / 54	<0.1 / 0.3	23
γ-Hexachlorocyclohexane	0.055	110 (±7.7)	56 (±3.8)	<0.1	16	0.55	65 / 75	0.5 / 0.5	23
Heptachlor	0.047	89 (±1.3)	76 (±1.5)	87 (±2.3)	16	0.47	89 / 98	89 / 93	23
Aldrin	0.038	87 (±1.6)	82 (±0.7)	92 (±1.9)	16	0.38	91 / 98	92 / 93	23
Heptachlor epoxide	0.029	79 (±1.3)	89 (±0.6)	92 (±2.0)	16	0.29	88 / 96	89 / 91	23
t-Chlordane	0.024	92 (±2.1)	100 (±4.9)	95 (±2.2)	16	0.24	91 / 98	92 / 94	23
c-Chlordane	0.031	90 (±2.2)	100 (±3.7)	96 (±2.1)	16	0.31	93 / 99	94 / 94	23
t-Nonachlor	0.030	87 (±2.0)	96 (±2.6)	95 (±1.5)	16	0.30	94 / 100	95 / 96	23
Dieldrin	0.038	84 (±2.1)	98 (±1.0)	93 (±1.6)	16	0.38	91 / 98	91 / 93	23
8-Monohydromirex	0.035	94 (±1.3)	99 (±1.8)	100 (±1.4)	16	0.35	101 / 100	95 / 97	23
10-Monohydromirex	0.033	85 (±1.2)	108 (±2.6)	96 (±1.7)	16	0.33	93 / 98	94 / 96	23
Mirex	0.039	92 (±1.7)	101 (±0.9)	95 (±1.9)	16	0.39	105 / 91	87 / 100	23
p,p'-DDT[f]	0.030	107 (±5.7)	90 (±5.6)	42 (±17.)	23	—	—	—	—
p,p'-DDE[g]	0.030	93 (±0.9)	95 (±1.5)	146 (±1.0)	23	0.30	94 / 101	95 / 96	23
p,p'-DDD[h]	0.062	105 (±3.4)	84 (±3.0)	51 (±5.4)	23	0.62	88 / 97	50 / 52	23
p,p'-DDMU[i]	—	—	—	32 (±1.4)	23	—	—	—	—
3,4,2',4',5'-Pentachlorobiphenyl[j]	2.2	99 (±1.7)	96 (±1.2)	91 (±0.2)	52	—	—	—	—
2,3,4,2',4',5'-Hexachlorobiphenyl[j]	2.8	92 (±1.6)	96 (±1.9)	95 (±3.1)	52	—	—	—	—
2,4,5,2',3',4',5'-Heptachlorobiphenyl[j]	3.9	90 (±1.6)	100 (±2.6)	99 (±0.7)	52	—	—	—	—

[a]All organochlorine compounds from 1,2,3-trichlorobenzene to p,p'-DDMU inclusive were eluted from the Florisil column as a single fraction with 250 mL 50% (v/v) CH2Cl2–hexane. The Aroclor and low-level p,p'-DDT, DDE, and DDD recoveries were determined in separate spiking experiments. Recoveries for freeze-dried samples were calculated relative to wet sample analyses in all cases, not the nominal spiking level.
[b]Mean ± CV (%), n = 4.
[c]Duplicate analyses.
[d]Samples (6 g) freeze-dried in a 6 cm diameter aluminum dish, 0.25 cm depth, except low level DDT, DDD, and DDE samples, which were processed as described in Footnote e.
[e]Samples (45–50 g) freeze-dried in a 250 mL beaker, 2 cm depth.
[f]1,1-Bis (p-chlorophenyl)-2,2,2-trichloroethane
[g]1,1-Bis(p-chlorophenyl)-2.2-dichloroethylene
[h]1,1-Bis(p-chlorophenyl)-2 2-dichloroethane
[i]1,1-Bis(p-chlorophenyl)-2-chloroethylene. This compound was formed by dehydrochlorination of p,p'-DDD in the freeze-dried, low-level sample only. Recovery based on p,p'-DDD equivalent weight.
[j]Based on percentage of peak 4 (50% (w/w) mixture of Aroclors 1254 and 1260).

program were therefore available to test the stability of the major organochlorine residues over a relatively long period in wet, frozen egg homogenate. In addition to these experiments, we report results on the effect of freeze-drying on organochlorine residue levels in whole-body herring gull homogenates. This experiment was preliminary to a study on long-term preservation in substrates other than eggs.

Experimental

Apparatus and Reagents

(a) *Gas chromatograph.*—Hewlett-Packard Model 5840A equipped with ^{63}Ni detector, splitless injector, and Model 7671A automatic sampler. Operating conditions: helium carrier gas inlet pressure 1.75 kg/sq. cm; 95% (v/v) argon–methane detector purge gas flow of 40 mL/min; injector temperature 250°C, detector temperature 300°C, initial oven temperature of 100°C, hold for 2 min, 10°C/min for 5 min, then 3°C/min to end of run (final temperature 300°C) for Fraction 1. Fractions 2 and 3 used similar programs with a rate of 5°C/min from 7 min to the end of the run.

(b) *Column.*—60 m × 0.147 mm id fused silica capillary column, DB-5 liquid phase, 0.1 μm film thickness (J&W Scientific).

(c) *Homogenizer.*—Sorvall Omni-Mixer Model 17150 with 400 mL stainless steel chamber assembly.

(d) *Freeze-dryer.*—Labconco Freeze-Dryer-12 with Virtis shelf cabinet (Labconco Corp., Kansas City, MO 64132).

Table 2. Effect of freeze-drying on residue levels in a pooled herring gull egg homogenate (duplicate analyses)

Compound	Residue level, mg/kg fresh wt		% Change after freeze-drying[a]
	Wet sample	Freeze-dried sample	
Hexachlorobenzene	0.063 0.067	0.057 0.058	−12
p,p'-DDE	5.2 5.0	4.7 4.6	−9
3,4,2',4',5',- Pentachlorobiphenyl[b]	0.93 0.89	0.85 0.83	−7
2,3,4,2',4',5'- Hexachlorobiphenyl[b]	1.8 1.8	1.7 1.7	−7
2,4,5,2',3',4',5'- Heptachlorobiphenyl[b]	0.93 0.89	0.86 0.84	−7
Mirex	0.15 0.16	0.16 0.15	<5
Oxychlordane	0.21 0.22	0.21 0.21	<5
c-Chlordane	0.014 0.016	0.018 0.016	+10
c-Nonachlor	0.10 0.11	0.11 0.10	<5
Heptachlor epoxide	0.12 0.11	0.11 0.11	<5
Dieldrin	0.28 0.28	0.29 0.27	<5

[a] A cut-off point of <5% was chosen because this is within the coefficient of variation of replicate injections.
[b] See also Figure 1.

Table 3. Effect of storage time on residue levels in a freeze-dried pooled herring gull egg homogenate stored at −18° to −28°C in the dark (duplicate analyses)

Compound	Residue level, mg/kg fresh wt after storage time of:			
	3 Months	6 Months	9 Months	12 Months
Hexachlorobenzene	0.059 0.058	0.062 0.062	0.062 0.064	0.064 0.065
p,p'-DDE	4.6 4.8	4.9 5.2	4.8 4.7	4.7 5.0
3,4,2',4',5'- Pentachlorobiphenyl[a]	0.87 0.87	0.96 1.0	0.97 0.93	0.93 1.0
2,3,4,2',4',5'- Hexachlorobiphenyl[a]	1.7 1.7	1.9 2.1	1.9 1.8	1.8 1.9
2,4,5,2',3',4',5'- Heptachlorobiphenyl[a]	0.9 0.9	1.0 1.2	1.0 1.0	1.0 1.1
Mirex	0.16 0.16	0.16 0.17	0.16 0.16	—[b] 0.18
Oxychlordane	0.20 0.20	0.21 0.21	0.21 0.21	0.21 0.21
c-Chlordane	0.018 0.018	0.019 0.020	0.020 0.018	0.020 0.020
c-Nonachlor	0.11 0.11	0.11 0.11	0.11 0.11	0.12 0.11
Heptachlor epoxide	0.12 0.12	0.12 0.12	0.12 0.11	0.11 0.11
Dieldrin	0.29 0.26	0.27 0.29	0.27 0.26	0.27 0.27

[a] See also Figure 1.
[b] Sample lost.

(e) *Glass mortar and pestle.*

(f) *Extraction and chromatographic columns.*—2.1 × 35 cm, glass with Teflon stopcock.

(g) *Rotary evaporator.*—Büchi Rotavapor-R with water bath temperatrue 33°C.

(h) *Glass evaporating flasks.*—Round with flat bottom, 250 mL and 500 mL with ⊤ 24/40 outer joint.

(i) *Roller culture apparatus.*—(Wheaton Scientific, or equivalent.)

(j) *Florisil.*—60–100 mesh PR grade (Floridin Co.). Transfer 750–800 g Florisil to porcelain dish and place in muffle furnace 6 h at 600°C. Reduce temperature to 130°C overnight. Transfer Florisil to brown glass bottle, and let cool with lid closed. Add 1.2% (v/w) water with graduated pipet and close bottle with Teflon-lined lid. Shake contents to break up lumps and transfer container to roller and roll slowly for 24 h to equilibrate before use.

(k) *Sodium sulfate.*—Anhydrous, granular (Fisher Scientific). Heat overnight in 400°C muffle furnace, cool, wash with hexane, and air-dry before use.

(l) *Glass wool.*—Wash with 50% (v/v) methylene chloride–hexane, drain solvent, air-dry, then heat at 400°C at least 1 h before use.

(m) *Solvents.*—Hexane, methylene chloride, methanol, and chloroform, distilled in glass (Caledon, Georgetown, Ontario, Canada).

(n) *Ultra centrifugal mill.*—Retsch Type ZM1 (Brinkmann Instruments, Rexdale, Ontario).

(o) *Homogenizer meat grinder.*—Model FW 70 (Bizerba).

(p) *Gel permeation chromatography apparatus.*—GPC Autoprep 1002A system (Analytical Bio-Chemistry Laboratories, Inc.).

Sample Preparation and Freeze-Drying

A large pool (8.2 kg) was made of naturally contaminated herring gull eggs from Lake Huron in 1980. Portions of this

pool or chicken eggs to be spiked (150–200 g) were homogenized in a 400 mL stainless steel container 20 min at low speed.

The pesticide spiking solution in hexane was added to the 400 mL stainless steel container and evaporated to 1–2 mL with a stream of nitrogen. The chicken egg homogenate (100 g) was then added to the solution and mixed 20 min at low speed. Table 1 gives compounds and artificial contamination levels. PCBs were spiked as a 50% (w/w) mixture of Aroclors 1254 and 1260.

Homogenates of whole-body herring gulls, which had been dosed intraperitoneally for a clearance rate experiment with hexachlorobenzene, γ-hexachlorocyclohexane, octachlorostyrene, oxychlordane, p,p'-DDE, 8-monohydromirex, and mirex, were prepared by passing the sample several times through a homogenizer meat grinder. The whole bird, including feathers, head, feet, and wings, was homogenized.

Preparation for freeze-drying the egg homogenates and whole body homogenate of experimentally dosed birds was performed in the following ways:

(A) Artificially contaminated chicken egg, "not stored," results in Table 1: A 6 g spiked chicken egg homogenate was placed on a 6 cm diameter aluminum dish to 0.25 cm depth, except for low level PCB, DDT, DDD, and DDE-contaminated samples, which were processed as in (B).

(B) Artificially contaminated chicken egg, "stored," results in Table 1: A 50 g spiked chicken egg homogenate was placed in a 250 mL beaker of 6.5 cm diameter to 2 cm depth.

(C) Pooled herring gull egg homogenate, results in Table 2, 3, and 4: A 150 g herring gull egg homogenate was placed in a 250 mL glass jar of 7 cm diameter to a 4.5 cm depth.

(D) Whole body herring gull homogenates, results in Table 6: A 100 g whole-body herring gull homogenate was spread in an aluminum dish to a 0.5 cm depth.

Samples (A) to (D) were allowed to freeze at −30°C overnight. Frozen samples were then freeze-dried 24 h with a

Table 4.   Effect of storage time on residue levels in a freeze-dried pooled herring gull egg homogenate stored at 23°C in the dark (duplicate analyses)

Compound	Residue level, mg/kg fresh wt after storage time of:			
	3 Months	6 Months	9 Months	12 Months
Hexachlorobenzene	0.059	0.059	0.064	0.063
	0.058	0.059	0.062	0.063
p,p'-DDE	4.6	4.5	5.0	4.7
	4.6	4.5	4.6	4.6
3,4,2',4',5'-Pentachlorobiphenyl[a]	0.85	0.85	0.99	0.96
	0.85	0.86	0.92	0.93
2,3,4,2',4',5'-Hexachlorobiphenyl[a]	1.7	1.7	1.9	1.9
	1.7	1.7	1.8	1.8
2,4,5,2',3',4',5'-Heptachlorobiphenyl[a]	0.86	0.85	1.1	1.02
	0.86	0.87	0.97	0.98
Mirex	0.15	0.16	0.16	0.17
	0.15	0.16	0.16	0.17
Oxychlordane	0.17	0.16	0.15	0.14
	0.17	0.16	0.16	0.14
c-Chlordane	0.018	0.020	0.018	0.021
	0.018	0.019	0.020	0.019
c-Nonachlor	0.10	0.11	0.11	0.11
	0.10	0.11	0.11	0.11
Heptachlor epoxide	0.12	0.11	0.12	0.12
	0.11	0.12	0.12	0.12
Dieldrin	0.26	0.24	0.27	0.26
	0.24	0.30	0.25	0.27

[a] See also Figure 1.

system vacuum of 1.4×10^{-4} kg/sq. cm and starting shelf temperature of $-30°C$. The shelf temperature controller was shut off at the beginning of the 24 h freeze-drying period, allowing the temperature to rise slowly to room temperature (25°C). The freeze-dried egg samples were transferred to a mortar, ground to a fine powder, and stored in tightly capped sample bottles either at room temperature in the dark or in a freezer at $-18°$ to $-28°C$. The freeze-dried whole-body herring gull homogenates were further ground with an ultracentrifugal mill to pass a 1.0 mm particle-size screen.

Analysis

Wet sample egg homogenates (5 g), freeze-dried egg homogenates reconstituted to 5 g with water, and wet whole-body herring gull homogenates (7 g) were presoaked ½ h in hexane, ground with 30 g Na₂SO₄ in a mortar, and extracted in a column with 300 mL hexane at a rate of 5–10 mL/min. The hexane eluate was concentrated with a rotary evaporator to less than 5 mL in preparation for Florisil cleanup.

Egg extracts were cleaned up by column chromatography on 30 g Florisil deactivated with 1.2% (v/w) water. The columns were dry-packed and pre-wetted with hexane. After the extracts were rinsed onto the columns with 5–10 mL of the first eluant (hexane), the columns were eluted as follows: 150 mL hexane (fraction 1), 150 mL 15% (v/v) CH₂Cl₂–hexane (fraction 2), and 200 mL 50% (v/v) CH₂Cl₂–hexane (fraction 3). Each fraction was concentrated to about 1 mL with a rotary evaporator and diluted to a suitable volume for GC analysis.

Freeze-dried, powdered whole-body herring gull homogenates (10 g) were extracted without reconstitution of water or grinding with Na₂SO₄. The extraction was carried out in a column as before, but using a stronger solvent system, because total lipid determination was desired for other purposes in this experiment. Samples in the column were pre-wetted ½ h with 40 mL methanol, and eluted with 250 mL 50% (v/v) methanol/chloroform. The extracts were evaporated just to

dryness with a rotary evaporator, diluted to about 50 mL with chloroform, and washed with 300 mL water. The washed chloroform extracts were dried by passing through a bed of Na₂SO₄, evaporated to dryness with a rotary evaporator for gravimetric determination of lipid, and then diluted to 20 mL with chloroform for storage before cleanup.

Because column chromatographic separation of compounds was not required before analysis, freeze-dried whole-body herring gull extracts were cleaned up by gel permeation chromatography (GPC). A 0.5 mL aliquot of the extract in chloroform was evaporated to dryness in a nitrogen stream, redissolved in 50% (v/v) CH₂Cl₂–hexane, and diluted to 10 mL with the same solvent. A 5 mL portion was cleaned up by GPC using a 45 × 3 cm id glass column packed with 60 g Bio-Beads S-X3, 200–400 mesh. The column was eluted with 50% (v/v) CH₂Cl₂–hexane at a rate of 5.5 mL/min. The portion of eluate from 151 mL to 280 mL was collected and evaporated in the rotary evaporator to almost dryness and then diluted to suitable volume with hexane for GC analysis. This method was compared to the extraction and cleanup method used for egg and wet whole-body homogenates matrices by analysis of 3 whole-body samples using both methods. The mean differences for all residues were within the expected experimental error ($\pm 9\%$).

Peak areas and multiple external standards were used for quantitation. Representative penta-, hexa-, and heptachlorobiphenyl (PCB) congeners were quantified from known weight percentages in a standard mixture of 50% (w/w) Aroclors 1254 and 1260: 3,4,2',4',5' pentachlorobiphenyl (4.5%); 2,3,4,2',4',5'-hexachlorobiphenyl (7.8%); and 2,4,5,2',3',4',5'-heptachlorobiphenyl (5.7%).

Results and Discussion

Artificially Contaminated Chicken Eggs

To check the contamination levels and homogeneity of the chicken egg homogenate spiked with low levels of 19 organochlorine residues by the method described, 4 analyses were done on the wet sample. The compounds were present at 91% ± 10 of the nominal spiking level, with a mean SD of 2.4 from replicate analysis, indicating that the spiked chicken egg was homogeneous in the freeze-drying experiment (Table 1). Percent recoveries in the low- and high-level contamination freeze-dried samples were calculated relative to wet sample analyses in all cases, not the nominal spiking level, because of possible losses inherent in the spiking process.

Results from analyses of artificially contaminated chicken eggs immediately after being freeze-dried are given in Table 1 under the column "% recovered, dried, not stored." Compounds with retention times less than that of heptachlor were lost roughly in proportion to their vapor pressures. Recoveries were affected by the physical dimensions of the sample matrix during freeze-drying, as indicated by the higher recoveries of compounds with volatilities less than that of aldrin in the "stored" than in the "not-stored" sample (Table 1), particularly at low-level contamination. The exposed surface-to-volume ratio of the "stored" sample during freeze-drying was 8 times higher than that of the "not-stored" sample. Recoveries from the "stored" sample were acceptable for compounds with volatilities less than or equal to that of hexachlorobenzene.

Both isomers of hexachlorocyclohexane (HCH) were not only lost due to volatilization, but were decomposed completely in the freeze-dried samples after 16–23 days storage at room temperature. In a similar study, Hill and Smart (5) also reported complete loss of α- and γ-HCH in freeze-dried
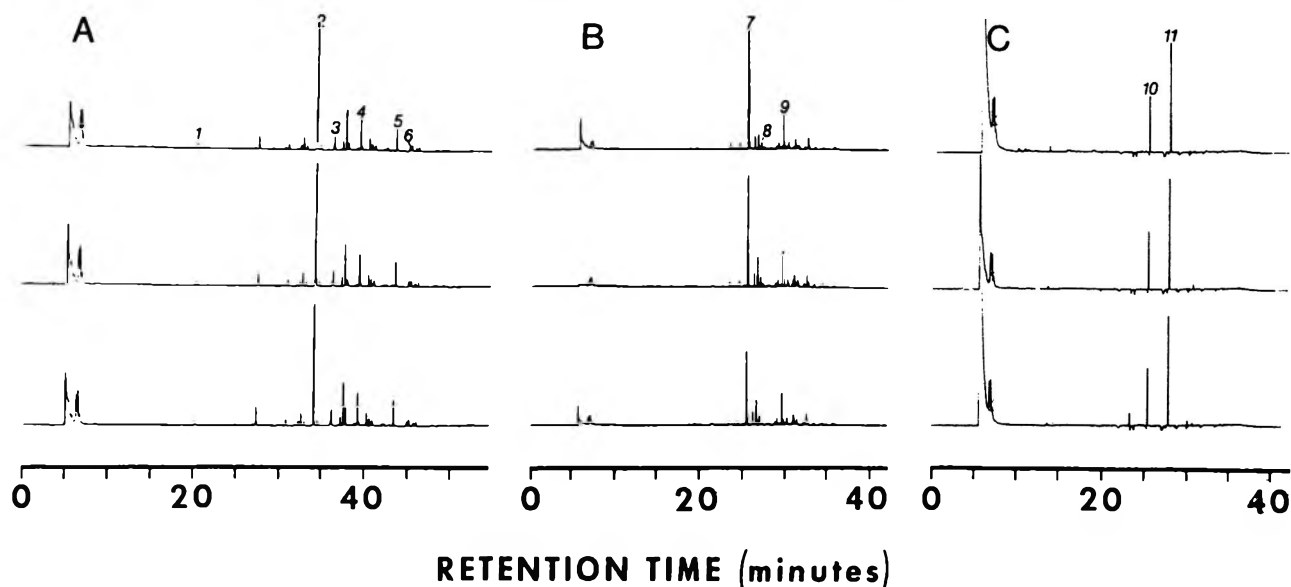
RETENTION TIME (minutes)

Figure 1.   Chromatograms of Florisil fractions of pooled naturally contaminated Lake Huron herring gull eggs, comparing organochlorine residues in the wet sample (top); freeze-dried sample stored at −18° to −28°C for 1 year (middle); freeze-dried sample stored at room temperature in the dark for 1 year (bottom).
Wet weight equivalent injected: A, fraction 1, 0.03 mg; B, fraction 2, 0.39 mg; C, fraction 3, 0.30 mg. Peaks are numbered as follows: (1) hexachlorobenzene; (2) p,p'-DDE; (3) 3,4,2',4',5'-pentachlorobiphenyl; (4) 2,3,4,-2',4',5'-hexachlorobiphenyl; (5) 2,4,5,2',3',4',5'-heptachlorobiphenyl; (6) mirex; (7) oxychlordane; (8) c-chlordane; (9) c-nonachlor; (10) heptachlor-epoxide; (11) dieldrin.

egg or egg fat matrices resulting from dehydrochlorination by a naturally occurring agent in the egg. Zabik (6) reported a 79% reduction cf lindane (γ-HCH) in whole egg and 49% in egg yolks after freeze-drying the samples. Storage time before analysis was not given.

As Hill and Smart (5) found, levels of p,p'-DDT and p,p'-DDD, both of which easily undergo dehydrochlorination to p,p'-DDE and p,p'-DDMU, respectively, decreased significantly in spiked chicken eggs after 11–23 days storage at room temperature. The decrease in p,p'-DDT and p,p'-DDD was quantitatively accounted for by the increase in p,p'-DDE and the appearance of p,p'-DDMU in the low-level sample. Because no p,p'-DDT existed in high-level contamination samples, no increase in p,p'-DDE was observed in these samples after storage. About 50% p,p'-DDD was lost after 23 days regardless of spiking level, but p,p'-DDMU was not found in the high-level sample. We have no explanation for this, except the possible effect of the higher level of other organochlorines on the p,p'-DDD decomposition mechanism.

Dehydrochlorination has been found to occur in other tissues. The half-life of p,p'-DDT in frozen avian liver at −12° to −15°C is about 65 days (7). Degradation of p,p'-DDT to p,p'-DDD probably occurs via reduced Fe-porphyrins because the reaction does not occur under aerobic conditions. Reductive dechlorination and dehydrochlorination of p,p'-DDT occurs in frozen whole blood also, with a half-life of about 4 weeks at −20°C (8).

Aldrin, heptachlor epoxide, trans-chlordane, cis-chlordane, trans-nonachlor, dieldrin, 8-monohydromirex, 10-monohydromirex, mirex, and PCB levels in the spiked chicken egg samples were not affected in any way by freeze-drying or storage at room temperature for short terms (15–52 days) after freeze-drying.

The conclusions on the effects of freeze-drying are therefore the same as those of Hill and Smart (5); easily dehydrochlorinated compounds such as hexachlorocyclohexane isomers, p,p'-DDD, and p,p'-DDT are lost rapidly in freeze-dried egg matrices. In addition, we have shown that signifi-

cant losses can occur during freeze-drying of compounds that have retention times on a capillary column less than heptachlor. Nonetheless, recoveries of even the most volatile constituent studied, 1,2,3-trichlorobenzene, did not appear to change after 23 days storage at room temperature. Even for relatively volatile organochlorines, volatilization losses after freeze-drying are therefore insignificant for short terms (e.g., 1 month), although significant losses may occur over several months or years for such compounds. Losses of organochlorine compounds with relative retention times greater than heptachlor (as a general indication of volatility) are therefore expected to be negligible over the long term. Oxychlordane is the only exception we have found to this rule (see next section).

Naturally Contaminated Herring Gull Eggs

Table 2 gives results from analysis of herring gull eggs immediately after freeze-drying. Of the compounds that were determined in this sample, apparently only hexachlorobenzene (HCB) was affected; the difference in levels between the wet and freeze-dried samples is not statistically significant because of the low concentration. The levels of p,p'-DDT and p,p'-DDD were too low in this sample for accurate determination; therefore, the dehydrochlorination that occurred in fortified chicken eggs could not be tested. The slight (10%) increase in cis-chlordane is not significant at the 0.01–0.02 mg/kg level.

A portion of the freeze-dried homogenate was stored at −18° to −28°C in the dark and analyzed for residue levels at 3-month intervals for 1 year (Table 3). PCB levels apparently increased slightly, which probably resulted from gradual changes in chromatographic separation over the period of analysis. There was no significant change in levels of any of the other organochlorine compounds after 1 year storage.

The results for room temperature storage (Table 4) were similar to those for −18° to −28°C storage, except that oxychlordane levels decreased by about 19% over 3 months and 14% more after 1 year for a total loss of 33%. We have shown similar losses for vacuum oven-drying of herring gull egg

Table 5. Trends of organochlorine residue levels in naturally contaminated herring gull egg homogenates stored at −18° to −28°C and analyzed periodically over a 2–3 year period[a]

Sample	Total storage, months	Heptachlor epoxide	Dieldrin	Oxy-chlordane	Hexachloro-benzene	p,p'-DDE	Mirex	PCB[b]
Lake Erie pool, 1979:								
Mean level (mg/kg) ± CV[c] (%)	36	0.12 ± 25	0.23 ± 9	0.13 ± 15	0.19 ± 16	4.2 ± 12	0.11 ± 17	82 ± 9
Trend during storage (%/y)[d]		+8.3	−5.7	−2.5[e]	−6.7	+9.7	−0.4	−6.7
Lake Huron pool, 1980:								
Mean level (mg/kg) ± CV[c] (%)	27	0.11 ± 19	0.28 ± 12	0.15 ± 14	0.077 ± 23	4.7 ± 10	0.14 ± 12	24 ± 16
Trend during storage (%/y)[d]		+4.2	−5.3	−7.4[f]	+5.3	+3.7	−6.4[f]	−12

[a]The pools were analyzed as blind reference samples in a quality assurance program. See also Figure 2.
[b]Calculated as a 50% (w/w) mixture of Aroclors 1254 and 1260 based on peak 4, Figure 1 (2,3,4,2',4',5'-hexachlorobiphenyl).
[c]Grand mean of all analyses. There were from 5 to 11 analyses done at each time point, for a total of 65 analyses of the 1979 pool and 84 analyses of the 1980 pool.
[d]The trends are calculated from linear regression of individual analyses against time. Lines are plotted in Figure 2.
[e]Total storage time of 24 months.
[f]Total storage time of 16 months.

Table 6. Effect of freeze-drying on residue levels in whole-body herring gull homogenate

	Group A			Group B			Group C		
Compound	Residue level in wet sample, mg/kg (mean ± CV)	No. of birds analyzed	% Change after freeze-drying (mean ± SD)	Residue level in wet sample, mg/kg (mean ± CV)	No. of birds analyzed	% Change after freeze-drying (mean ± SD)	Residue level in wet sample, mg/kg (mean ± CV)	No. of birds analyzed	% Change after freeze-drying (mean ± SD)
Hexachlorobenzene	2.8 ± 9%	4	−4 ± 8	4.4 ± 23%	6	−18 ± 10	5.7 ± 14%	6	−16 ± 10
γ-Hexachlorocyclohexane	—	—	—	—	—	—	3.1 ± 18%	6	−18 ± 6
Octachlorostyrene	2.7 ± 5%	4	−14 ± 4	4.4 ± 23%	6	−13 ± 3	6.1 ± 13%	6	−8 ± 5
Oxychlordane	0.52 ± 27%	4	−22 ± 8	4.4 ± 21%	6	−20 ± 9	5.4 ± 14%	6	−11 ± 5
p,p'-DDE	7.2 ± 21%	4	−24 ± 5	6.8 ± 21%	6	−26 ± 4	7.7 ± 14%	6	−13 ± 5
8-Monohydromirex	5.5 ± 23%	4	−14 ± 3	5.9 ± 22%	6	−13 ± 4	6.9 ± 12%	6	−5 ± 4
Mirex	4.9 ± 27%	4	−10 ± 5	5.4 ± 19%	6	−10 ± 3	6.2 ± 11%	6	−3 ± 4
Percent lipid	23.0 ± 4.0%	4	+0.9 ± 0.7	18.7 ± 4.6%	6	+0.3 ± 1.3	18.8 ± 2.6%	6	+0.8 ± 1.1

homogenates at 40–50°C. It is, therefore, possible that the losses of oxychlordane at room temperature in freeze-dried eggs result from volatilization. HCB was lost, however, to the same extent as oxychlordane during vacuum oven-drying, whereas no HCB was lost during storage of the freeze-dried homogenate at room temperature. Therefore, chemical decomposition of oxychlordane is probably occurring at room temperature. The present study cannot confirm this because no decomposition products were identified. It is interesting to note that heptachlor epoxide, which differs from oxychlordane only by having one fewer chlorine (at the 2-position on one side of the epoxide bridge), is completely stable under all treatments.

Figure 1 shows chromatograms of each Florisil column fraction, for comparison of the wet sample residue levels to those remaining after 1 year under the 2 storage regimes. The decrease in oxychlordane is obvious in the room temperature storage chromatogram. A small peak, which may be an oxychlordane decomposition product, appeared in the third fraction of this sample.

Long-term stability of many organochlorine residues in fresh, frozen herring gull eggs can be estimated from analyses of 2 egg pools (Lake Erie, 1979; and Lake Huron, 1980). Inserted as blind reference samples over a 3-year period, from 1979 to 1982, these pools were part of a quality assurance program in routine analysis of herring gull eggs from the Great Lakes. The samples (6–10 g) were archived individually in glass scintillation vials with aluminum foil-lined caps, at temperatures of −18° to −28°C. The results are summarized in Table 5 and plotted in Figure 2. Linear regression of residue level vs. time over a 2–3 year period showed statistically significant slopes in several cases, but the apparent trends were often up as well as down. The percent changes per year

predicted by linear regression were similar in magnitude to the CV for replicate determinations at any time point (6–10%) and are, therefore, not significant. Changes in analytical methodology and variation in the quality of analysis probably accounted for most of the trends. For example, capillary gas chromatography was introduced between 6 and 12 months on the scale in Figure 2. Anas (9) found no indication of loss of total DDT plus PCBs in 3 seal blubber samples re-analyzed after 2 years frozen storage.

Because the data were obtained in a blind manner in which a number of important variables (such as methodology and equipment) were not controlled, they represent a "worst case" situation for frozen egg homogenates and lend confidence to the simple deep-freezing technique for preservation of organochlorine compounds in this substrate over periods of years. The real loss rate per year is probably much less than 10% for any compound studied on the basis of data for freeze-dried samples (Tables 3 and 4) obtained under controlled conditions. This conclusion may not apply to other tissues or whole-body homogenates; therefore, a separate long-term storage project is being developed to test the stability of organochlorine compounds in whole, frozen, non-egg substrates.

Whole-body Herring Gull Homogenates

Table 6 gives results from the analysis of wet and freeze-dried whole-body homogenates of experimental gulls. Lipid recovery from the freeze-dried sample was marginally higher than that from the wet sample in most cases, but the differences were not significant. In general, organochlorine compounds were lost to a greater extent from whole body homogenates than they were from egg homogenates, but the degree of loss was highly variable, ranging from 3.2% for mirex in
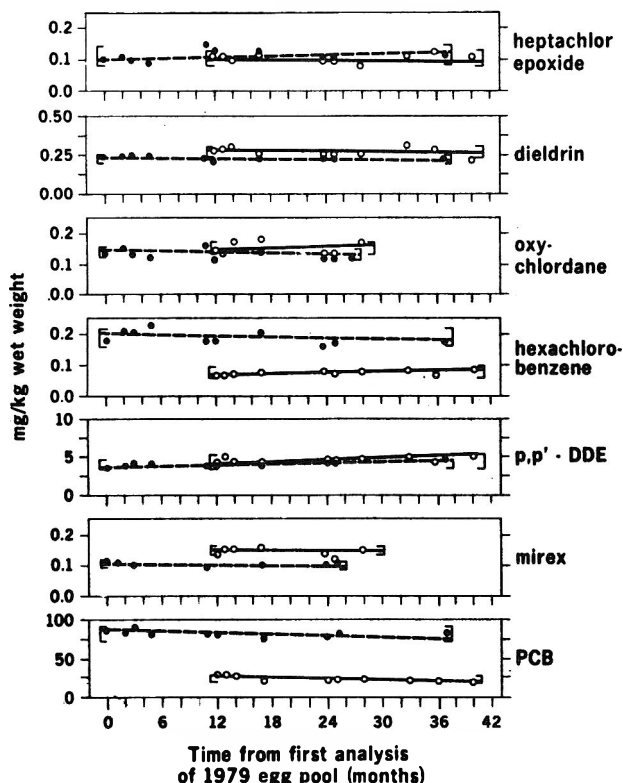
Figure 2. Trends of organochlorine residue levels in naturally contaminated herring gull egg homogenates stored at −18° to −28°C: filled circles and dashed lines, Lake Erie pool, 1979; open circles and solid line, Lake Huron pool, 1980.

Each point represents the mean of 5–11 analyses for a total of 65 analyses of the 1979 pool and 84 analyses of the 1980 pool. Data were obtained from a quality assurance program. The lines are based on linear regressions of the whole data set for each residue. The open-sided boxes at the end of each line represent the range for ± 1 SD from the grand mean of the data set. PCBs are calculated as a 50% (w/w) mixture of Aroclors 1254 and 1260 based on peak 4, Figure 1 (2,3,4,2′,4′,5′-hexachlorobiphenyl).

Group C to 25.9% for p,p′-DDE in Group B. Except in Group C, volatility of the compound appeared to play less of a role than did other factors, because relatively involatile compounds such as p,p′-DDE and 8-monohydromirex were lost to a similar or greater extent than hexachlorobenzene and γ-hexachlorocyclohexane. The exposed surface-to-volume ratio during freeze-drying was high because the samples were spread out thinly to hasten the rate of water loss. Organochlorines may have co-distilled with the water vapor. Losses during evaporation of the chloroform–methanol extract to dryness twice before analysis could have accounted for lower levels in the freeze-dried sample relative to the wet sample, which was in hexane and never taken to dryness, however, re-analysis of 3 freeze-dried samples using the procedure for wet sample analysis indicated the same levels by both procedures. Conditions for freeze-drying of tissue homogenates should be carefully designed to prevent losses by using low surface-to-volume ratios for the samples, even if the time to complete water removal is increased.

General Conclusions and Recommendations

Freeze-drying did not affect the levels of most organochlorine residues in egg substrates, except for compounds more volatile than hexachlorobenzene. Losses by vaporization from the sample occurred principally during the freeze-drying process itself, rather than during subsequent storage. The main advantage of freeze-drying is that it opens the possibility of inexpensive, long-term storage at room temperature without decomposition of the sample. Indications are that some compounds (p,p′-DDT, p,p′-DDD and hexachlorocyclohexane isomers in this study) are subject to dehydrochlorination under these conditions. Oxychlordane was gradually lost. Because it is difficult to predict a priori which compounds might be labile, or how the sample may be used after several years storage, freeze-drying cannot be recommended as a generally applicable method for long-term preservation of egg substrates for subsequent organochlorine analysis. Changes in residue levels appeared to be relatively unaffected by 3-year storage of wet samples at −18° to −28°C, and this method of preservation is therefore recommended. Very low temperatures (liquid nitrogen, −196°C) probably will not offer any advantage, and may be much more expensive to operate, but as a precautionary measure, a temperature of −30°C is preferable to −18°C to preserve the sample matrix from bacterial/enzymatic degradation. It should be cautioned that no conclusions can be drawn from the present study about dehydrochlorination in samples frozen in fresh condition because the long-term storage samples contained no easily dehydrochlorinated compounds.

Less extensive data were obtained on whole-body tissue homogenates. Generally, freeze-drying produced higher residue losses in whole-body homogenates than in egg homogenates, but the freeze-drying conditions were not optimum for minimizing losses resulting from volatilization or co-distillation. A project to study the effect of long-term storage of frozen tissue homogenates on residue levels has been initiated. Until more data are obtained, we recommend low-temperature storage of tissue homogenates over freeze-drying.

REFERENCES

(1) Luepke, N.-P. (Ed.) (1979) Proceedings of the International Workshop, Berlin (West), Oct. 23–28, 1978, Martinus Nijhoff Publishers, The Hague, The Netherlands
(2) Elliott, J. E. (1982) International Workshop on Environmental Specimen Banking and Monitoring as Related to Banking, Saarbrüken, FRG, May 10–15, 1982
(3) Kawar, N. S., De Batista, G. C., & Gunther. F. A. (1973) Residue Rev. 48, 45–77
(4) Egli, H. (1982) J. Agric. Food Chem. 30, 861–866
(5) Hill, A. R., & Smart, N. A. (1981) J. Agric. Food Chem. 29, 675–679
(6) Zabik, M. E. (1971) J. Food Sci. 36, 87–88
(7) Jefferies, D. J., & Walker, C. H. (1966) Nature 212, 533–534
(8) Ecobichon, D. J., & Saschenbrecker, P. W. (1967) Science 156, 663–665
(9) Anas, R. E. (1974) Pestic. Monit. J. 8, 12–14

MYCOTOXINS

Determination of Aflatoxins in Vegetable Oils

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A simple method is proposed for determination of aflatoxins in vegetable oils. The method was successfully applied to both crude and degummed oils. The oil sample, dissolved in hexane, was applied to a silica column and washed with ether, toluene, and chloroform; aflatoxins were eluted from the column with chloroform–methanol (97 + 3). As quantitated by thin layer chromatography and liquid chromatography, the oils analyzed contained aflatoxin B_1 at levels of 5–200 µg/kg. Recoveries of aflatoxin B_1 standards added to aflatoxin-free oils were between 89.5 and 93.5%, with coefficients of variation of 6.3–8.0%.

The presence of aflatoxins in peanuts is a well known problem worldwide (1). Although various separation and sorting systems have been developed to remove infested kernels, many batches of peanuts—and, consequently, oils—are still heavily contaminated with aflatoxins. A rapid method to indicate aflatoxin in vegetable oils is therefore needed.

The determination of aflatoxins in vegetable oils is usually based on partition between 2 immiscible solvents. Aflatoxin is extracted from the oil into a polar solvent and subsequently partitioned into chloroform (2–4). Although the recoveries are acceptable, these methods are time consuming, require large quantities of solvents, and frequently involve troublesome emulsions.

A simple method for determination of aflatoxins in vegetable oils was developed which is suitable for thin layer chromatography (TLC), as well as for liquid chromatography (LC) quantitation, and is a convenient alternative to the liquid partition methods.

METHOD

Apparatus

Equipment specified is not restrictive; other suitable equipment can be substituted.

(a) *Liquid chromatograph.*—Varian 5000 with 10 µL Valco loop injector; Milton Roy LDC UV III Monitor detector with 360 µm lamp and filter, 0.004 AUFS sensitivity setting; Hewlett-Packard 3390A recorder, set to peak integration mode.

(b) *LC column.*—Stainless steel, 25 cm × 4.6 mm id, packed with 5 µm spherical silica gel (Nucleosil 50-5).

(c) *Cleanup column.*—Borosilicate glass, 200 × 20 mm id, fitted with sintered-glass discs, taps, and 100 mL reservoirs.

Reagents

(a) *Solvents.*—AR grade methanol, hexane, and toluene. Acetonitrile, chloroform, cyclohexane, and 2-propanol, distilled in glass. Ethyl ether, distill in glass and pass through neutral aluminum oxide before use. Water, distilled.

(b) *LC mobile phase.*—Fraction A: $CHCl_3$–cyclohexane (800 + 240). Fraction B: acetonitrile–2-propanol–water (32 + 22 + 0.7). Mix fractions A and B well by shaking, and filter under reduced pressure through 0.6 µm membrane filter before use.

(c) *Silica gel for column chromatography.*—E. Merck silica gel 60, 70–230 mesh (No. 7734). Activate by drying 2 h at

105°C, add water at 1 mL/100 g, seal, mix well, and equilibrate overnight in airtight container (5).

(d) *Anhydrous sodium sulfate.*—AR grade.

(e) *TLC plates.*—E. Merck silica gel 60 without fluorescence indicator, layer thickness 0.25 mm (No. 5721).

(f) *Aflatoxin standards.*—Prepare individual aflatoxin stock solutions, and determine concentration by UV absorbance (6). For LC determinations, prepare working standard in chloroform containing 1 ng B_1/µL. For TLC determination, dilute small volumes of above working standard with equal volumes of methanol to produce standard containing 0.5 ng/µL. Discard this solution after 1 week.

Sample Preparation

Mix oil vigorously until uniform sample is obtained. If necessary, heat to 40–50°C to ensure homogeneity. Immediately weigh 20 g sample into 100 mL glass beaker, add 20 mL hexane, and mix thoroughly.

Column Chromatography

Weigh 20 g silica gel into 100 mL beaker. Add 50 mL hexane and stir thoroughly to remove air. Slowly transfer slurry to glass column with aid of more hexane, let settle, and add enough Na_2SO_4 to give 2 cm upper layer. Drain solvent, letting some hexane remain above Na_2SO_4. Transfer diluted sample to column, rinse beaker with hexane, and add rinse to column. Let sample percolate through column. Wash successively with 100 mL hexane, 100 mL ethyl ether, 20 mL toluene, and 100 mL chloroform. Elute aflatoxin with 200 mL chloroform–methanol (97 + 3) at ca 5 mL/min. Evaporate to near dryness under reduced pressure at 40–50°C. Transfer residue to small amber vial, using several small portions of chloroform, and evaporate chloroform under stream of nitrogen just to dryness. Dissolve residue in 200–1000 µL chloroform, depending on amount of aflatoxin present in sample. Stopper securely to prevent evaporation losses.

Thin Layer Chromatography

Spot TLC plates, prepared according to AOAC (7), with aliquots of samples, together with aflatoxin standards. Develop plates in 2 dimensions, using chloroform–acetone (9 + 1) and ethyl ether–methanol–water (96 + 3 + 1) in unsaturated tanks. Determine amount of aflatoxin in sample test spot by viewing under 360 nm UV light and comparing with standard aflatoxin spots. For optimum visual quantitation, test spot should contain between 1 and 2 ng aflatoxin, and be same size as standard spot or spots (see *Results and Discussion*).

LC Determination

Set mobile phase flow rate to 1.4 mL/min. Under this condition, 1 ng/µL of aflatoxin B_1 elutes after 5 min and gives peak height equivalent of 70% FSD. Peak area responses are linear over a range of 0.05–2.0 ng/µL, and resolution of aflatoxins B_1, B_2, G_1, and G_2 is adequate. From either integrator area count or peak height measured tangentially to apex of peak, aflatoxin B_1 is calculated as follows:

$$B_1, (µg/kg) = P \times C \times D/P' \times W$$

Table 1.   Recovery of aflatoxin B₁ added to degummed sunflower oil[a]

Added, µg/kg	LC, peak area		LC, peak height		Av. rec., %	CV, %
	Found	Rec., %	Found	Rec., %		
5	4.9	98	4.3	86	93.5	6.3
	4.95	99	4.4	88		
	4.95	99	4.55	91		
10	8.7	87	8.0	80	89.5	8.0
	9.8	98	8.0	90		
	8.6	86	8.0	80		
	9.5	95	8.2	82		
	10.2	102	9.4	94		
	9.4	94	8.6	86		
50	46.5	93	43.0	86	90	7.2
	46.0	92	40.5	81		
	50	100	44	88		

[a]Data represent average values of all area and peak height measurements.

Table 2.   Aflatoxin in crude peanut oil (day-to-day variation)

Sample	Found, µg/kg
1	220
2	200
3	230
4	200
Av.	212.5
CV, %	7.1

where P = area or peak height of sample; P' = area or peak height of standard; C = concentration of aflatoxin B₁ in standard, ng/µL; D = dilution of sample extract; W = weight of sample, g. Measurement by integrated peak areas gives slightly better recoveries than does peak height measurement.

Results and Discussion

Two sets of vegetable oils were tested: degummed sunflower oil and crude peanut oil. Twenty g samples of an aflatoxin-free sunflower oil were spiked at 5, 10, and 50 µg aflatoxin B₁/kg and analyzed according to the above method. Final determinations were made by LC; results are presented in Table 1. Recoveries were calculated from both peak heights and peak areas. Peak height measurements usually gave lower recoveries than did area measurements. Data represent the averages of peak height and area recoveries.

The crude peanut oil was analyzed on different days over a period of 2 weeks. Aflatoxins were quantitated by LC and TLC; results are presented in Table 2.

LC determination of aflatoxin B₁ gave good results on spiked, degummed oils. For crude peanut oil containing more than 100 µg/kg, LC results were also satisfactory, largely because

the extract was diluted and artifact peaks were consequently negligible. Lower concentrations of aflatoxin in crude oil could be determined by TLC, but further cleanup was required for LC analysis.

Careful adjustment of the aliquots spotted on TLC plates allowed quantitation comparable to LC. The use of a standard of 0.5 ng aflatoxin B₁/µL chloroform–methanol (1 + 1) is advantageous because the polar nature of the solvent allows aflatoxin to diffuse over the whole area of the standard spot during application. With care and experience, the standard can be spotted so that, after development in the second direction, the resultant spots are of identical size, resulting in more accurate quantitation.

The proposed column chromatographic method is reliable and efficient, and isolation of aflatoxins from vegetable oils can be accomplished in one step.

REFERENCES

(1) Mirocha, C. J., Pathre, S. V., & Christensen, C. M. (1980) in *Advances in Cereal Science and Technology*, Y. Pomeranz (Ed.), American Assoc. of Cereal Chemists, Inc., St. Paul, MN, Vol. 3, Chapter 5, pp. 159–225

(2) Parker, W. A., & Melnick, D. (1966) *J. Am. Oil Chem. Soc.* **43**, 635–638

(3) Robertson, J. A., Lee, L. S., Cucullu, A. F., & Goldblatt, L. A. (1965) *J. Am. Oil Chem. Soc.* **42**, 467–471

(4) Velan, M., & Reynaud, J. (1967) *Rev. Fr. Corps Gras* **14**(5), 305–310

(5) *Official Methods of Analysis* (1984) 14th Ed., AOAC, Arlington, VA, Sec. **26.052–26.060**

(6) *Official Methods of Analysis* (1984) 14th Ed., AOAC, Arlington, VA, Sec. **26.006–26.011**

(7) *Official Methods of Analysis* (1984) 14th Ed., AOAC, Arlington, VA, Sec. **26.074**

PESTICIDE FORMULATIONS

Reverse Phase Liquid Chromatographic Determination of Edifenphos in Technical and Emulsifiable Concentrate Formulations

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A convenient reverse phase liquid chromatographic method has been developed for determination of edifenphos in its technical product and emulsifiable concentrate. The method allows determination of the active ingredient of the formulation directly, without any solvent extraction. In addition, reference standard edifenphos has been obtained by preparative liquid chromatography.

Edifenphos *(O*-ethyl *S,S*-diphenyl phosphorodithioate), an important agricultural fungicide, has high prophylactic activity against *Pyricularia oryzae;* its emulsifiable concentrate formulation is used throughout the world for control of rice blast disease (1). Two analytical methods are commonly used for analysis of the technical product (2): In the iodometric titration, the total thiophenol content is first estimated by titrating with iodine solution. The content of free thiophenol, pre-determined similarly, is subtracted from the total thiophenol evolved from a known quantity of edifenphos technical. This method did not give repeatable results, probably because the end point determination is not very sharp. The second method, gas chromatography (GC), uses an internal standard measurement of the area under each peak. Edifenphos is a very high boiling liquid, which decomposes during distillation at atmospheric pressure. Therefore, its stability under GC conditions is doubtful.

Currently, liquid chromatography (LC) is assuming importance in pesticide analysis. We explored this technique for determination of edifenphos technical and formulations.

Experimental

Apparatus

Liquid chromatograph.—Spectra-Physics, Model 8000B, equipped with pump, UV-visible variable wavelength detector (Model 4000B), connected to loop injection system, printer/plotter-type recorder, and microprocessor controlled data system.

Reagents

(a) *Solvents for chromatography.*—Double-distilled water and distilled acetonitrile (guaranteed 99.8% pure). Equal volumes of both solvents (freshly distilled) were mixed and degassed by stirring, and then filtered through Whatman paper No. 42.

(b) *Reagents for iodometric titration.*—1N H_2SO_4; $NaHCO_3$; NaOH; 0.1N iodine; 0.1% phenolphthalein indicator solution in ethanol.

(c) *Edifenphos.*—Hinosan® E.C. (manufacturing date March 22, 1983, batch No. 3/8, marketed by M/S Bayer (India) Ltd., label of 25% surfactant and 50% edifenphos by weight.

Chromatographic conditions:

(a) For analytical
Column packing   Lichrosorb® RP-8

Column dimensions	250 mm × 4.6 mm
Column temperature	ambient
Mobile phase	acetonitrile–water (1 + 1)
Flow rate	2 mL/min
Sample size	10 μL
Detector	UV, 254 nm at sensitivity of 0.08 AUFS
Chart speed	1 cm/min

(b) For preparative

Column packing	Lichrosorb® RP-8
Column dimensions	250 mm × 10 mm
Column temperature	ambient
Mobile phase	acetonitrile–water (1 + 3)
Flow rate	5 mL/min
Sample size	500 μL
Detector	UV, 254 nm at sensitivity of 0.16 AUFS
Chart speed	1 cm/min

Identity Experiments

We prepared 2.5 mg technical sample of edifenphos by condensing *O*-ethyl phosphorodichloridate with thiophenol in the presence of sodium ethoxide (1). This was dissolved in 25 mL acetonitrile–water (1 + 1) and 10 μL was injected and chromatographed according to the analytical conditions described above. At constant pressure mode of operation of the pump, 3 peaks were recorded at 557 psi. The percent of each component calculated by the respective peak area (Figure 1) was 1 min (6%, I), 4.4 min (8.7%, II, thiophenol), and 8.7 min (85%, III, edifenphos).

Preparation of Standard

A high purity standard of edifenphos was not readily available. We obtained a pure sample from the available technical material (88% pure) by preparative LC. A solution of 50 mg technical edifenphos in 10 mL acetonitrile–water (1 + 3) was injected (500 μL/injection) and chromatographed according to the conditions described above. Fractions corresponding to the main peak at 33 min were collected, and the whole process was repeated 20 times. All fractions were combined and extracted twice with 75 mL benzene. The benzene extract was dried over anhydrous sodium sulfate (5 g) and distilled. After the last traces of solvent were removed under vacuum, the residue was weighed carefully (4.6 mg), dissolved in 50 mL solvent mixture, and 10 μL was injected onto the analytical column. A single peak was obtained (Figure 2). The identity of the compound was also checked by infrared and nuclear magnetic resonance spectroscopy.

Analysis of Technical Samples and Calculations

Standard reference edifenphos of known concentration was first injected and chromatographed for calibration purposes. Three batches of technical edifenphos were chromato-
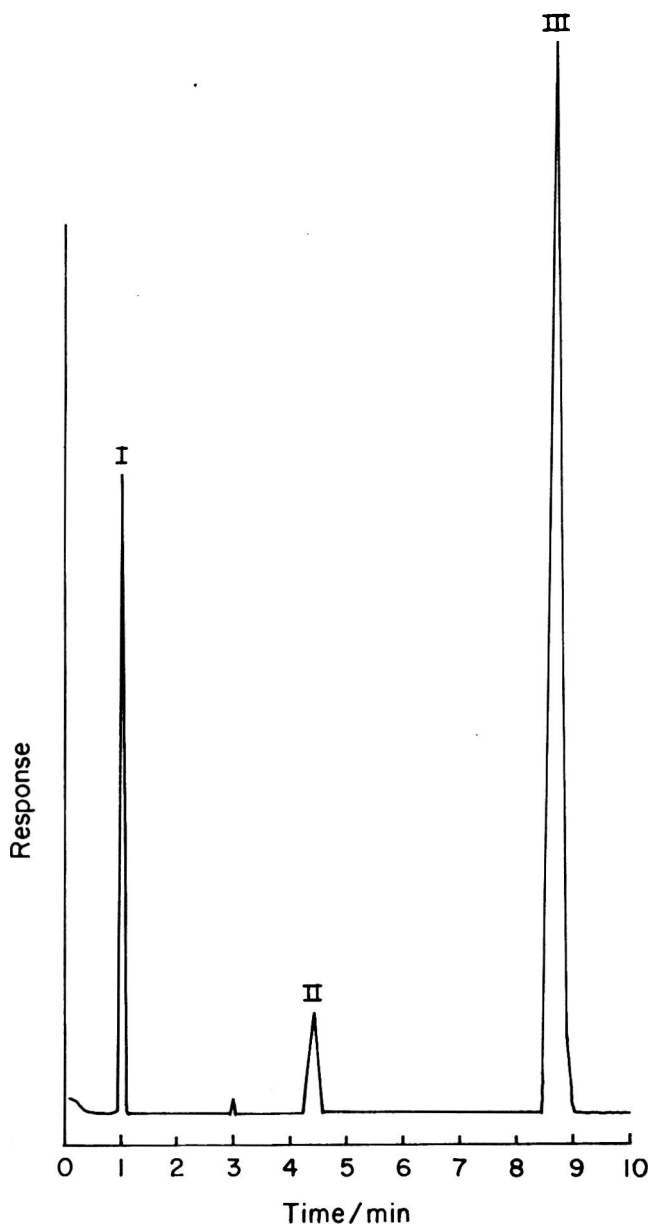
Figure 1. Analytical chromatogram of edifenphos (tech.): I, unidentified; II, thiophenol; III, edifenphos. See text for chromatographic conditions.
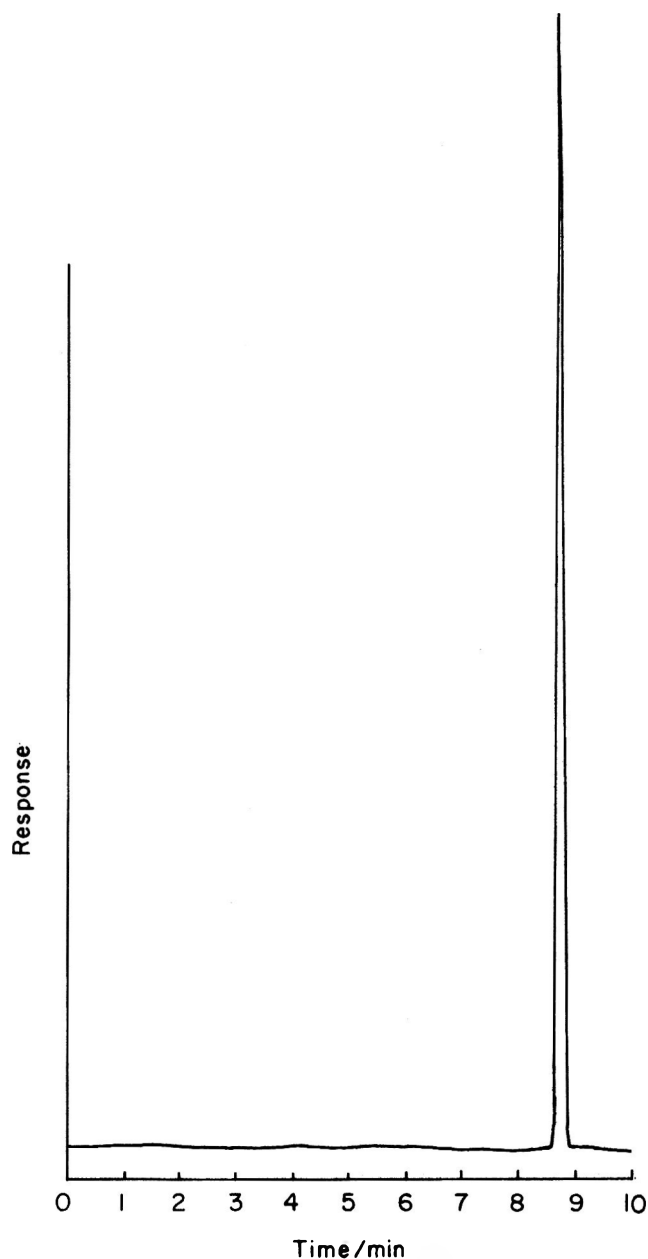

Figure 2. Analytical chromatogram of pure edifenphos. See text for chromatographic conditions.

graphed, and their purity was calculated by comparing the area of the relevant peak with that of the standard (3) as follows:

$$\text{Concn} = \text{area}/K_f$$

where concn = concentration of the component (ppm); area = area of the peak corresponding to the component; and K_f = area of standard/concn of standard (ppm).

Analysis of Technical Samples with Iodometric Titration

To enable comparison with the LC method, all the above technical samples of edifenphos were analyzed by iodometric titration as follows:

Determination of total thiophenol.—To 0.5 g sample dissolved in 30 mL ethanol, 5 g NaOH and 20 mL water were added. The mixture was refluxed 4 h under nitrogen atmosphere, cooled to room temperature, and diluted to 250 mL, using, first, 90 mL ethanol and then distilled water. Fifty mL of this solution was mixed with excess 1N H_2SO_4 and 0.1 g $NaHCO_3$ and then titrated with 0.1N I_2 solution.

Determination of free thiophenol.—To 0.5 g sample dissolved in 40 mL ice-cooled ethanol, 20 mL cold water, 5 mL 1N H_2SO_4, and 0.5 g $NaHCO_3$ were added. This was titrated with 0.1N I_2 solution. Edifenphos content was calculated as follows:

Percentage of edifenphos by mass =
$$[(5A/M_1 - B/M_2)] \times 1.552 \times 0.935$$

where A and B are the volumes in mL of iodine solution consumed for total thiophenol and free thiophenol determination, respectively; M_1 and M_2 are masses in g of samples taken for total thiophenol and free thiophenol determinations respectively.

Analysis of Formulated Samples

Two samples of emulsifiable concentrates, one prepared in the laboratory by the standard procedure and the other procured commercially, were analyzed. The laboratory formulation was prepared by combining 25% Tween-80 and 50% edifenphos by weight. For the commercial sample, 0.5 g was

Table 1. Accuracy and precision of results obtained by liquid chromatography and comparison with iodometric method for determining edifenphos

Result	Technical			50% E.C.	
	I	II	III	Laboratory	Commercial
Concn % by iodometric method (n = 3)	87.6	88.7	88.8	—[a]	—
Concn % by LC method (n = 4)	88.59	89.65	89.47	50.05	48.47
Std dev.	1.67	1.45	0.953	0.58	1.06
t-Value (P = 0.05)	1.18	1.310	1.40	0.17	-2.88
95% Conf. interval	2.65	2.30	1.51	0.93	-1.68

[a]None determined.
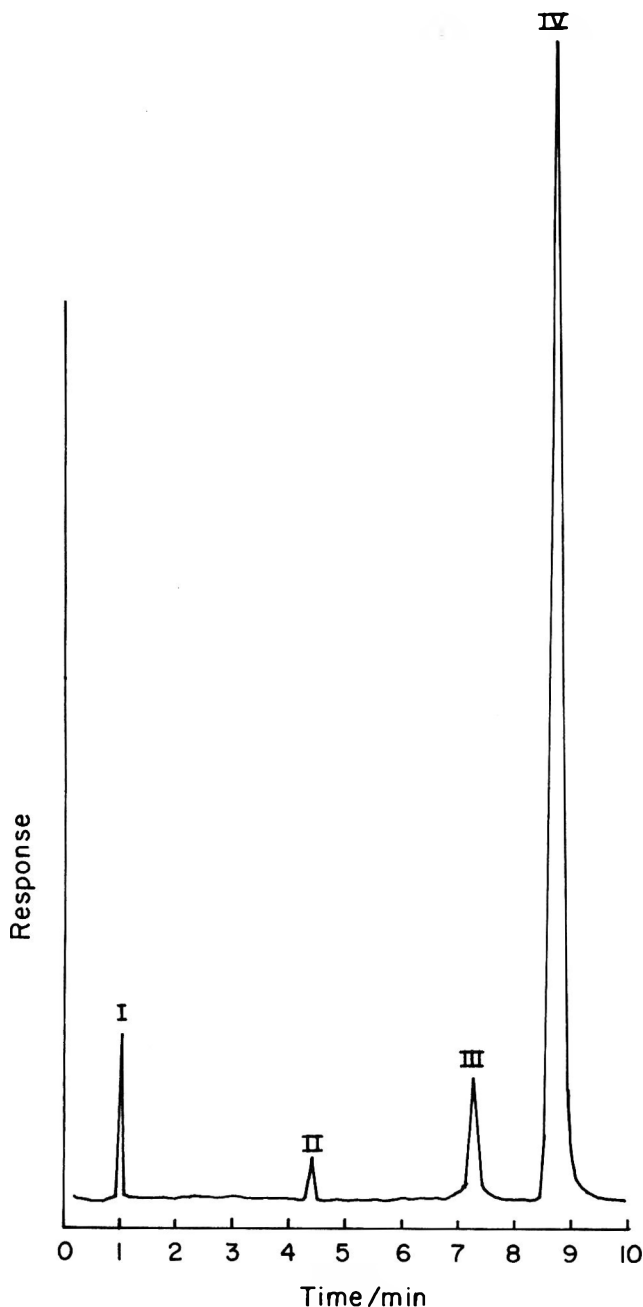
Figure 3. Analytical chromatogram of edifenphos emulsifiable concentrate: I, unidentified; II, thiophenol; III, extra peak not present in technical; IV, edifenphos. See text for chromatographic conditions.

dissolved in 50 mL solvent mixture; 1 mL of this solution was further diluted to 50 mL. After filtering through Whatman paper No. 42, 10 μL solution was injected onto the analytical column (Figure 3). Standard edifenphos (5 mg/50 mL) solution was also injected. The active ingredient of both samples was calculated by comparing the areas of the standard and samples.

Results and Discussion

A typical chromatogram of technical edifenphos is shown in Figure 1. Apart from the major peak of edifenphos (III), there are 2 minor peaks. The peak at 4.4 min (II) was attributed to thiophenol by comparison with an authentic sample. Other compounds that could be formed during edifenphos synthesis or storage are the corresponding diphenyl disulfide and ethyl phosphoric acid. Authentic samples of these compounds were compared under similar chromatographic conditions, but none corresponded to peak I.

The method of obtaining standard edifenphos by preparative LC is more convenient and reliable than the earlier method: repeated crystallization of the technical material with a mixture of toluene–n-hexane (1 + 1) and then with ethyl ether at −20°C. Our sample had a purity of 99.8% (Figure 2) and was used as an external reference standard for determination of active ingredient in all unknown samples.

Results of analysis by LC for 3 different technical samples have been compared with those by iodometric titration (Table 1). Analysis by Student's t-test (4) revealed no significant difference between results obtained by the 2 methods. The 95% confidence interval for emulsifiable concentrates by LC was ±2.65%.

Various impurities present in different batches of technical samples were also analyzed; the maximum amount of thiophenol was 5.45% and that of unidentified impurity, 5.93%.

Sensitivity of this method was tested; samples containing up to 0.5 ppm active ingredient can be satisfactorily detected at 0.04 AUFS.

The present LC method has many advantages over the GC method: First, no internal standard is needed. Second, the analysis is carried out at ambient temperature, so there is no chemical transformation or degradation resulting from heat. Last, the formulation can be conveniently analyzed by direct injection after dilution with solvent mixture, without interference from surfactants.

Acknowledgment

References

(1) Eto, M. (1974) *Organophosphorus Pesticides, Organic and Biological Chemistry*, CRC Press, Boca Raton, FL, pp. 34, 319

(2) Indian Standard Specification No. IS:8954-1978, Indian Standard Institution, Manak Bhavan, New Delhi, India

(3) Instrumental Manual, SP8000 Data System, Spectra-Physics, Berkeley, CA pp.7-31

(4) Snedecor, G. W. (1956) *Statistical Methods: Applied to Experiments in Agriculture and Biology*, 5th Ed., Iowa State University Press, Ames, IA pp.45

TECHNICAL COMMUNICATIONS

Determination of Mass per Unit Volume, Alcohol, Reducing Sugars, Total Acids, and Volatile Acids in Wine by the Methods of the International Office of Vine and Wine: Summary of Collaborative Study

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Original Publication: Bull. OIV (1982) **621**, 774–800
Methods:

Mass per unit volume at 20°C by pycnometer; OIV A1

Alcohol: distillation and determination of mass per unit volume by pycnometer on distillate; OIV A2

Reducing sugars: Luff-Schoorl; reduction of alkaline citrate solution and iodometric titration of unreduced copper; OIV A4C (usual method)

Total acidity: direct titration to pH 7 (not including CO_2 or SO_2); OIV A10 (usual method)

Volatile acidity: steam distillation from acid solution and titration (not including CO_2 or SO_2); OIV A11

Experimental Design: Two studies	1979–1980	1980–1981
No. of laboratories	13	11
No. of wines	3	3
No. of replicates	5	5

Statistical Analysis: By ISO 5725, calculating the following data:

mean ± standard deviation for each laboratory for each wine;

within-laboratory (S_0) and among-laboratories (S_x) standard deviations obtained by 1-way analysis of variance, material by material;

ISO repeatbility ($r = 2\sqrt{2}S_0$) and reproducibility ($R = 2\sqrt{2}S_x$);

standard deviations converted to corresponding coefficients of variation (CV) for this summary by multiplying by 100/mean.

Outlier Treatment: Within-laboratory: Check the 5 replicate values by the Grubbs test at the 95% confidence level (CL); if the critical value is exceeded, make 3 additional determinations. Check the 8 values by the Grubbs test at the 99% CL; if the critical value is exceeded, omit the extreme value from the calculations, but report it.

Among-laboratories: Check variance among laboratories by the Bartlett test at the 95% CL; if homogeneous, conduct analysis of variance of the laboratory averages, using a 99% CL for the test of significance. If the variances are not homogeneous, apply the Cochran maximum variance test. If the Cochran statistic is exceeded, eliminate the laboratory.

These rules may be bypassed for practical reasons: when too many laboratories must be eliminated so that the remaining number is no longer representative; if inquiry as to the source of systematic error will require too much work; or when a relatively large among-laboratories reproducibility can be tolerated. Therefore, some laboratories are still included which contain systematic errors.

Performance Parameters: See Table 1.

Comments by William Horwitz

The interlaboratory precision of the results for the determination of alcohol in wine by distillation and determination of the mass per unit volume (density) is the best of the almost

500 collaborative studies which have been examined thus far in a research program that is examining method performance. The coefficient of variation among-laboratories (CV_x) is about 0.5%. An analyst would expect duplicates to agree (repeatability, *r*) within about 0.1% alcohol (absolute) and to agree with another analyst in another laboratory (reproducibility, *R*) to about 0.2% alcohol (absolute), 19 times in 20, for an alcohol content of 10 to 17%. The direct determination of the mass per unit volume of wine by the pycnometer method shows even better precision:

	Within-lab.	Among-labs
CV (rel.)	0.004%	0.015%
Prediction interval (absolute)	0.001 (*r*)	0.0004 (*R*)

However, the precision of a physical measurement cannot be compared directly with the precision of a chemical measurement, because the latter varies considerably with concentration, which has no counterpart in physical measurements.

The results for the determination of reducing sugars and total acidity are likewise excellent, with CV_x between 1 and 2%, at concentration levels between 0.4 and 10%. The lowest reducing sugar value for the dry red wine, 0.2%, has a relatively high CV_x of 6%, compared to the other values. It is exhibiting the phenomenon of an exponential increase in CV_x, which is relatively constant over almost 2 orders of magnitude, until the method approaches its limit of determination. Where this cut-off point is taken depends on how much error can be tolerated at the low level. If a reducing sugar value of 1.0% distinguishes a dry wine from a "soft" wine, a reproducibility of 0.1% ($CV_x = 10\%$) might be tolerated; but if the cut-off is 0.5%, a reproducibility of 0.03% ($CV_x = 6\%$) may not be good enough. The function of the laboratory, in this respect, is to indicate how much uncertainty exists as a result of laboratory operations. The function of administrators is to determine how much uncertainty they will tolerate before triggering legal or economic sanctions.

Another interesting aspect of this study is the substantial number of outlying laboratories that had to be removed, because of aberrant results, from these classical determinations in food analysis. These outliers involved both displacement of means (systematic error) from most of the other mean values and an unusually wide range of values within a laboratory. The outliers were negligible only for the alcohol determination. In the other determinations, from 1 to 4, but usually 2 laboratories (about 17%), were removed by the administrator as outliers from the analyses of each material. Fewer outliers appeared in the second study than in the first. Laboratories 2, 6, and 13, all of which produced a substantial number of outliers in the first study, did not take part in the second study. Laboratories tend to do their best work when participating in collaborative studies. They are able to see only their repeatability during and immediately after the study.

Table 1. Precision parameters for important methods of analysis for wines as determined in 2 collaborative studies conducted by the International Office of Vine and Wine (only in the case of mass per unit volume was it necessary to differentiate between sweet and other wines[a])

Wine	Mean	Original No. of labs	Outlying labs removed No.	%	Repeatability	CV₀	Reproducibility R	CVₓ	Ratio R/r

Analysis: Mass Per Unit Volume at 20°C by Pycnometer   Method: OIV A1

Wine	Mean	Orig. labs	No.	%	r	CV₀	R	CVₓ	R/r
1979 study									
Red	0.99339	13	1	8	0.00009(48)	0.003	0.00029(59)	0.010	3.2
Soft rose	1.00257	13	2	15	0.00012(44)	0.004	0.00036(54)	0.013	3.0
Sweet white	1.03286	13	3	23	0.00020(40)	0.007	0.00053(49)	0.018	2.7
1980 study									
Soft rose	1.00030	10	3	30	0.00005(28)	0.002	0.00045(34)	0.016	9.0
Soft white	0.99977	10	4	40	0.00005(24)	0.002	0.00031(29)	0.011	6.2
Sweet white	1.02049	11	1	9	0.00016(42)	0.005	0.00043(51)	0.015	2.7
Average or (total)									
Dry and soft wines	0.99901	(46)	(10)	22	0.00008	0.003	0.00035	0.013	5.4
Sweet wines	1.02668	(24)	(4)	17	0.00018	0.006	0.00048	0.017	2.7

Analysis: Alcohol Content (% by volume)   Method: OIV A2 (distillation and pycnometer)

Wine	Mean	Orig. labs	No.	%	r	CV₀	R	CVₓ	R/r
1979 study									
Red	10.91	13	0	0	0.09 (52)	0.3	0.20 (64)	0.6	2.2
Soft rose	10.55	13	0	0	0.12 (57)	0.4	0.17 (69)	0.6	1.4
Sweet white	15.41	13	1	8	0.10 (52)	0.2	0.23 (63)	0.5	2.3
1980 study									
Soft rose	11.58	11	1	9	0.05 (40)	0.2	0.15 (49)	0.5	3.1
Soft white	11.05	11	0	0	0.05 (44)	0.2	0.17 (54)	0.5	3.1
Sweet white	16.64	11	0	0	0.09 (44)	0.2	0.21 (54)	0.45	2.3
Average or (total)	12.69	(72)	(2)	3	0.08	0.25	0.19	0.5	2.4

Analysis: Reducing Sugars (g invert sugar/L)   Method: Luff-Schoorl, OIV A4C, Usual Reduction Method

Wine	Mean	Orig. labs	No.	%	r	CV₀	R	CVₓ	R/r
1979 study									
Red	1.88	13	2	15	0.09 (47)	1.7	0.33 (57)	6.2	3.7
Soft rose	26.39	13	2	15	0.42 (47)	0.6	1.33 (57)	1.8	3.2
Sweet white	118.6	13	3	23	1.81 (40)	0.5	6.96 (49)	2.1	3.8
1980 study									
Soft rose	24.66	11	2	18	0.40 (36)	0.6	0.79 (44)	1.1	2.1
Soft white	26.01	11	2	18	0.37 (36)	0.5	1.24 (44)	1.7	3.4
Sweet white	91.38	11	2	18	1.18 (39)	0.45	4.00 (47)	1.55	3.4
Average or (total)	25.69	(35)	(6)	17	0.39	0.6	1.12	1.5	2.9
Sweet wines only	105.0	(24)	(5)	21	1.49	0.5	5.48	1.8	3.6

Analysis: Total Acidity (mequiv./L)   Method: OIV A10, Usual Titration Method
(mequiv./L × 0.075 = g tartaric acid/L)

Wine	Mean	Orig. labs	No.	%	r	CV₀	R	CVₓ	R/r
1979 study									
Red	84.2	13	3	23	1.0 (40)	0.4	5.0 (49)	2.1	5.0
Soft rose	100.8	13	3	23	0.9 (40)	0.3	2.7 (49)	0.95	3.0
Sweet white	70.4	13	3	23	0.8 (40)	0.4	1.8 (49)	0.9	2.3
1980 study									
Soft rose	85.2	11	1	9	0.7 (40)	0.3	3.9 (49)	1.6	5.6
Soft white	81.1	11	0	0	0.9 (44)	0.4	3.6 (54)	1.6	4.0
Sweet white	55.9	11	0	0	1.1 (44)	0.7	3.2 (54)	2.0	2.9
Average or (total) (mequiv./L)	79.6	(72)	(10)	14	0.9	0.4	3.4	1.5	3.8
(g tartaric acid/L)	5.97				0.07		0.26		

Analysis: Volatile Acidity (mequiv./L)   Method: OIV A11 Distillation and Titration
(mequiv./L × 0.060 = g acetic acid/L)

Wine	Mean	Orig. labs	No.	%	r	CV₀	R	CVₓ	R/r
1979 study									
Red	9.7	13	4	31	0.8 (39)	2.8	1.5 (47)	5.5	1.9
Soft rose	5.4	13	3	23	0.6 (40)	3.8	1.0 (49)	6.6	1.7
Sweet white	6.9	13	3	23	0.8 (40)	4.0	1.4 (49)	7.4	1.8
1980 study									
Soft rose	7.4	11	2	18	0.5 (39)	2.5	1.3 (47)	6.1	2.6
Soft white	6.4	11	2	18	0.5 (36)	2.5	1.0 (44)	5.4	2.0
Sweet white	8.2	11	2	18	0.6 (36)	2.5	1.2 (44)	5.0	2.0
Average or (total) (mequiv./L)	7.3	(72)	(16)	22	0.6	3.0	1.2	6.0	2.0
(g acetic acid/L)	0.44				0.04		0.07		

[a] Numbers in parentheses associated with r and R are degrees of freedom; repeatabilities: $r = \overline{2}\, 2S_0$ (S_0 = within-laboratory standard deviation, CV_0 = within-laboratory coefficient of variation; reproducibilities: $R = 2\sqrt{2}S_x$ (S_x = among laboratories standard deviation); CV_x = among-laboratories coefficient of variation (r and R are in the same absolute units as the mean; CV_0 and CV_x are in % (relative)).
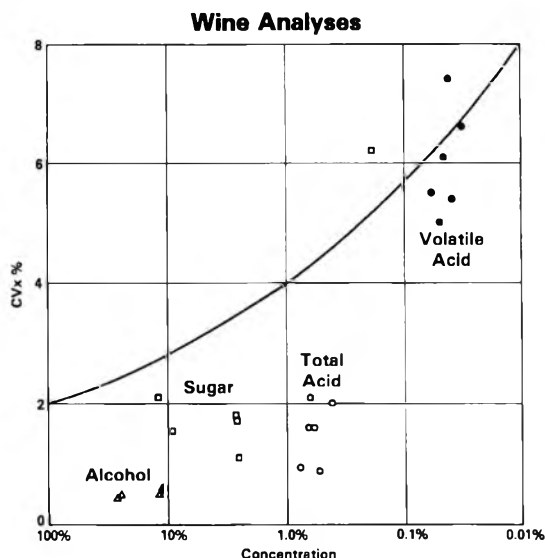
Wine Analyses

Figure 1.   The OIV precision data on wines with the general coefficient of variation (CV$_x$, %) vs log concentration curve (solid line, CV$_x$ = 2 exp (1 − 0.5 log C)). Key: x = alcohol, volume % (3% outliers removed); □ = reducing sugars, % invert sugars (17% outliers removed); ○ = total acidity, g tartaric acid/100 mL (14% outliers removed); • = volatile acidity, g acetic acid/100 mL (22% outliers removed).

If the repeatability is too great, the instructions directed that additional determinations be performed to reduce the standard deviation. But a systematic error of the mean is not apparent to a laboratory until all the results are reported. This explains why there are 4 times as many mean outliers as there are range outliers, and why, in all cases except the alcohol determination, the outliers approach a 20% value, which, on the basis of our experience, is excessive. These outlying laboratories do not do as good work as they think they do. The volatile acidity determination (0.04% as acetic acid), which is the lowest constituent concentration measured, had the highest CV$_x$ of about 6%, but only after a substantial fraction of the laboratories (almost 25%) were eliminated. This method, a steam distillation and titration to a phenolphthalein end point, has the greatest potential for variability and erratic performance. When very conservative outlier removal is practiced (in this case removing only 2 laboratories from analyses of 1 of the 6 materials), the CV$_x$ is about 11%, which is somewhat high for a 0.04% concentration (expected value about 6.5%).

Figure 1 gives the CV values that were accepted by the administrator of the study, plotted against the log concentration, and shows that almost all the values as good as or better than the curve representing the summary of all collaborative studies reviewed thus far.

Extraction of Organic Acids by Ion-Pair Formation with Tri-n-Octylamine. Part 7. Comparison of Methods for Extraction of Synthetic Dyes from Yogurt

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Synthetic dyes were extracted from yogurt by different methods, but all methods had in common a liberation of dyes from the food followed by ion-pair formation with tri-n-octylamine. Extraction with pH 5.5 phosphate buffer gave high recoveries for 5 of the 7 dyes investigated and was relatively fast. Precipitation of proteins followed by polyamide adsorption and desorption gave high yields for all the dyes but was tedious and long.

The use of additives in dairy products is very strictly regulated. Only a few additives are allowed in yogurt by the Belgian food legislation (1). Synthetic dyes are prohibited, which means that yogurt with fruit may be additionally colored only with natural dyes, such as beet red, cochineal, or carotenoids.

To verify compliance with the regulations, an analysis scheme for synthetic dyes was needed. In earlier papers (2–4), we described the determination of synthetic dyes in various foods by ion-pair extraction with tri-n-octylamine (TnOA). The same methodology was also applied for determination of benzoic acid, sorbic acid, and saccharin (5, 6).

As described before, in dye analysis, problems are often encountered because of high binding affinity of dyes to proteins (3, 4, 7). The major analytical problem consists then of liberating the dye from the bulk of the food matrix. In the past, we have tried to overcome this difficulty by including polyamide adsorption (4) or column elution with a methanol-ammonia mixture (3). Benzoic acid, sorbic acid, and saccharin were liberated from the yogurt matrix by extraction with pH 5.5 phosphate buffer (6). In the present work, a few

cleanup procedures were tested and compared for recovery, length of analysis, and ease of manipulations.

Experimental

Apparatus

(a) *Spectrophotometer.*—Perkin Elmer Hitachi 200.

(b) *pH Meter.*—Orion Ionalyser 601 and combined glass + calomel electrode.

Reagents

(a) *Tri-n-octylamine.*—Aldrich Europe (Beerse, Belgium).

(b) *Dyes.*—P. Entrop (Machelen, Belgium).

(c) *Polyamide for column chromatography.*—Woelm (Eschwege, GFR).

(d) *Buffer pH 5.5.*—For 2 L and ionic strength = 0.1: Dissolve 24.65 g NaH$_2$PO$_4$.H$_2$O and 1.26 g Na$_2$HPO$_4$.2H$_2$O in double-distilled water and dilute to 2 L with water.

(e) *Dye standard solutions.*—Dye solutions were prepared in a suitable solvent at 0–5 mg/mL, and further diluted with water to 0.1–0.5 mg/mL stock solutions.

(f) *Spiked samples.*—A 0.5 mL aliquot of dye stock solution was mixed with sufficient blank yogurt to produce 10 g mixture.

Extraction Methods

A. *Extraction with buffer.*—Transfer 0.5 g colored yogurt to glass, screw-cap centrifuge tube, and add 7 mL pH 5.5 phosphate buffer. Shake tube 20 min and centrifuge 10 min at 2000 rpm. Transfer supernatant liquid to 25 mL volumetric flask, and repeat extraction with phosphate buffer twice more.

Table 1. Comparison of methods for recovery of dyes extracted from yogurt (initial dye concentration 10 ppm)

Dye	Method recovery, %	
	Extn with buffer (A)	Pptn of proteins (B)
Tartrazine	96.4	93.3
Quinoline yellow	91.0	84.8
Sunset yellow	102.0	93.2
Azorubine	2.4	97.5
Amaranth	82.6	68.4
Cochenille red A	78.9	93.1
Erythrosine	0	70.5

Table 2. Recovery of dyes from yogurt as a function of dye concentration (extraction with buffer (A))

Dye	Concentration of dye, mg/kg				
	5	10	15	20	25
Tartrazine	88.1	96.4	91.3	85.1	88.8
Quinoline yellow	86.1	91.0	90.7	86.8	87.2
Sunset yellow	100.0	102.0	93.5	92.7	93.3
Azorubine	0	2.4	2.0	1.9	2.1
Amaranth	90.5	82.6	83.0	86.1	81.1
Cochenille red A	89.9±0.3	78.9±1.2	85.7±1.1	87.8±3.64	87.2±2.7
Erythrosine	0	0		0	0

Dilute combined extracts to 25 mL with phosphate buffer. This is extract A.

Extract 10 mL extract A with 10 mL 0.1M TnOA in chloroform; back-extract 5 mL chloroform with 5 mL 0.1M aqueous perchlorate. Determine dye content of this latter phase by photometry.

B. Precipitation of proteins.—Transfer 5 g colored yogurt to glass centrifuge tube, add 10 mL 5% ammonia, and shake tube vigorously 2 min. Add 10 mL acetone, shake tube 10 min, and then centrifuge 10 min at 2000 rpm. Store tube at 4°C for 2 h. Transfer supernatant solution to distillation flask and evaporate acetone at ca 60°C in rotary evaporator. Adjust pH of residual solution to 4.0 ± 0.1 with 30% phosphoric acid and transfer solution to glass centrifuge tube. Add 0.6 g polyamide and shake tube for 15 min. Centrifuge tube 10 min at 2000 rpm and discard supernatant liquid. Wash polyamide 3 times with ca 15 mL double-distilled water. Shake polyamide 15 min with 10 mL methanol–ammonia (95 + 5), and then centrifuge 10 min at 2000 rpm. Transfer methanol–ammonia to 25 mL volumetric flask, and repeat desorption with 10 mL of the same mixture. Dilute combined extracts to 25 mL with the same mixture, and measure dye content of methanol–ammonia solution by photometry.

Results and Discussion

All the extraction procedures described in this paper were tested only with yellow, orange, and red synthetic dyes; these colors occur most frequently in yogurt with fruit. The concentrations chosen produced realistically colored yogurts.

First, the simplest extraction with TnOA was tried, namely, addition of pH 5.5 buffer and subsequent partition to a chloroform phase containing TnOA. The recoveries obtained, however, were low (30–80%), and erythrosine was not extracted at all.

A previous paper from our laboratory described the ion-pair extraction with TnOA of sorbic acid, benzoic acid, and saccharin from yogurt with fruit (6). Before extraction, the additives were first liberated from the yogurt by repeated extraction with pH 5.5 phosphate buffer. This method quantitatively liberated the analytes investigated, so the same

methodology was tested for dyes. As shown in Table 1, the yield of this extraction is satisfactory and independent from the analyte concentration (Table 2). This efficient liberation of the dyes from the yogurt matrix results from the quantitative ion-pair formation with TnOA.

Azorubine and erythrosine are not liberated from yogurt. For these 2 dyes, the recoveries were measured in the chloroform phase because of their low back-extraction yield with perchlorate. Consequently, this method is satisfactory for tartrazine, quinoline yellow, sunset yellow, amaranth, and cochenille red A, but another analysis is needed for all synthetic dyes.

Previous extraction methods have been unsuccessful because of interaction of the dyes with the yogurt matrix (probably adsorption on the milk proteins). We sought to eliminate these interferences by either precipitation of the proteins or specific adsorption of the synthetic dyes on an adsorbent, e.g., polyamide. The first step, i.e., precipitation, was tried with acetone. Recovery of the dyes was very low, which confirms the result obtained previously with sorbic acid (6). Similar adsorption problems were encountered in the analysis of gelatin-containing sweets (4). In that case, dyes were first adsorbed on polyamide, the gelatin—which is also a protein—was washed away, and the extract was further purified by ion-pair extraction with TnOA. We tried this procedure with yogurt.

The pH of yogurt is acidic, and dyes are acids, so the addition of a base (e.g., ammonia) should increase the solubility of the dyes because of a higher degree of ionization. Therefore, the sample was treated with ammonia, mixed, centrifuged, and separated. This procedure liberated strongly adsorbed dyes, such as erythrosine, from the yogurt. The ammonia extract was acidified, polyamide was added, and the solution was mixed and centrifuged. The color of the polyamide indicates that not all dyes were adsorbed, although quantitative adsorption should have occurred (4). Clearly, this indicated that matrix constituents still influenced the extraction process.

We then tried to combine the alkaline medium (increasing solubility) with precipitation of proteins (eliminating interfering substances) by successively adding ammonia and acetone to yogurt. Table 1 shows that good recoveries were obtained for all dyes. Erythrosine yielded about 70% and azorubine recovery was also much higher than with previous methods.

To fully compare the results of methods A and B, one should bear in mind that recoveries are determined in a perchlorate phase for the former, and in methanol–ammonia for the latter. The usual determination procedure, as described for gelatin-containing sweets (4), identifies and quantitates the extracted dyes by liquid chromatography. This requires further sample treatment, i.e., extraction with TnOA and back-extraction with perchlorate, and involves additional losses. For tartrazine, quinoline yellow, sunset yellow, amaranth, and cochenille red A, these losses are only of the order of 1–2%, but for erythrosine and azorubine these losses are much higher due to an incomplete back-extraction. Extraction B, therefore, gives higher yields than extraction A.

In subsequent experiments, we tried to improve recoveries by making minor changes in the polyamide method; however, neither the use of acetonitrile instead of acetone nor the increased ammonia concentration had a significant effect on yields.

It was concluded that, in general, method B gives the best results. If the determination of tartrazine, quinoline yellow, sunset yellow, amaranth, and cochenille red A (alone or in a mixture) is of interest, procedure A is preferred because it

gives equally satisfactory recoveries but with less tedious manipulations.

Consequently, in the analysis of an unknown sample, method A should be tried first. Dyes are then identified and quantitated as described previously (2–4). If the sample remains red, which could indicate the presence of azorubine or erythrosine, then procedure B must be carried out as well. The extracted dye is then identified and quantitated as described before (4). If the yogurt is still red after ammonia–acetone treatment, this may indicate the presence of cochineal. Cochineal, a natural dye, is strongly adsorbed by yogurt constituents and is not liberated by any of the procedures used.
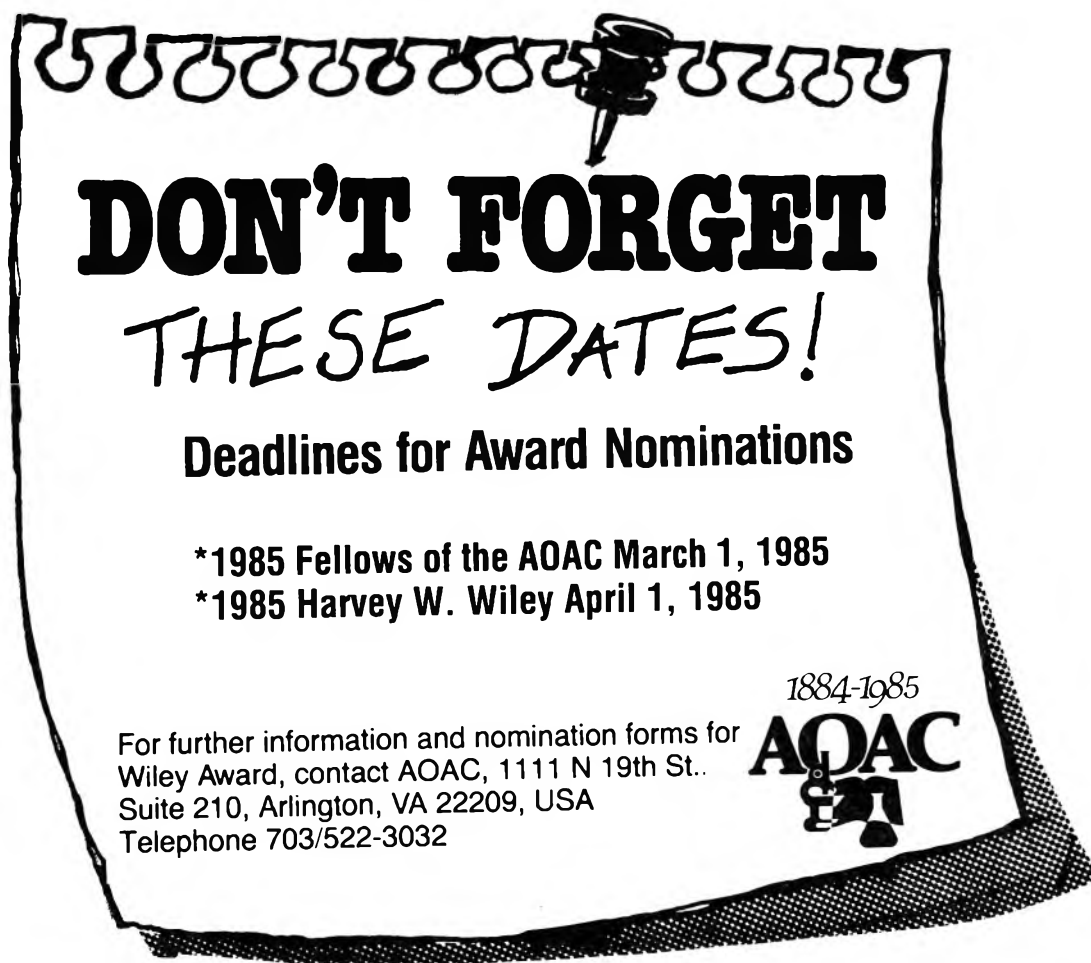
No synthetic dyes were found in the commercial yogurt with fruit samples that were already described in an earlier publication (5). Extractions were carried out with the buffer extraction method. Dyes were detected and measured by liquid chromatography.

REFERENCES

(1) Belgian Food Legislation KB 22/7/1982
(2) Puttemans, M.. Dryon, L., & Massart, D. L. (1982) *J. Assoc. Off. Anal. Chem.* **65**, 730–736
(3) Puttemans, M.. Dryon, L., & Massart. D. L. (1983) *J. Assoc. Off. Anal. Chem.* **66**, 670–672
(4) Puttemans, M.. Dryon, L., & Massart, D. L. (1983) *J. Assoc. Off. Anal. Chem.* **66**, 1039–1044
(5) Puttemans, M., Dryon L., & Massart, D. L. (1984) *J. Assoc. Off. Anal. Chem.* **67**, 880–885
(6) Puttemans, M., Branders, C., Dryon, L., & Massart, D. L. (1985) *J. Assoc. Off. Anal. Chem.* **68**, 000–000
(7) Lehmann, G.. Collet, P., Hahn, H. -G., & Ashworth, M. R. F. (1970) *J. Assoc Off. Anal. Chem.* **53**, 1182–1189

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