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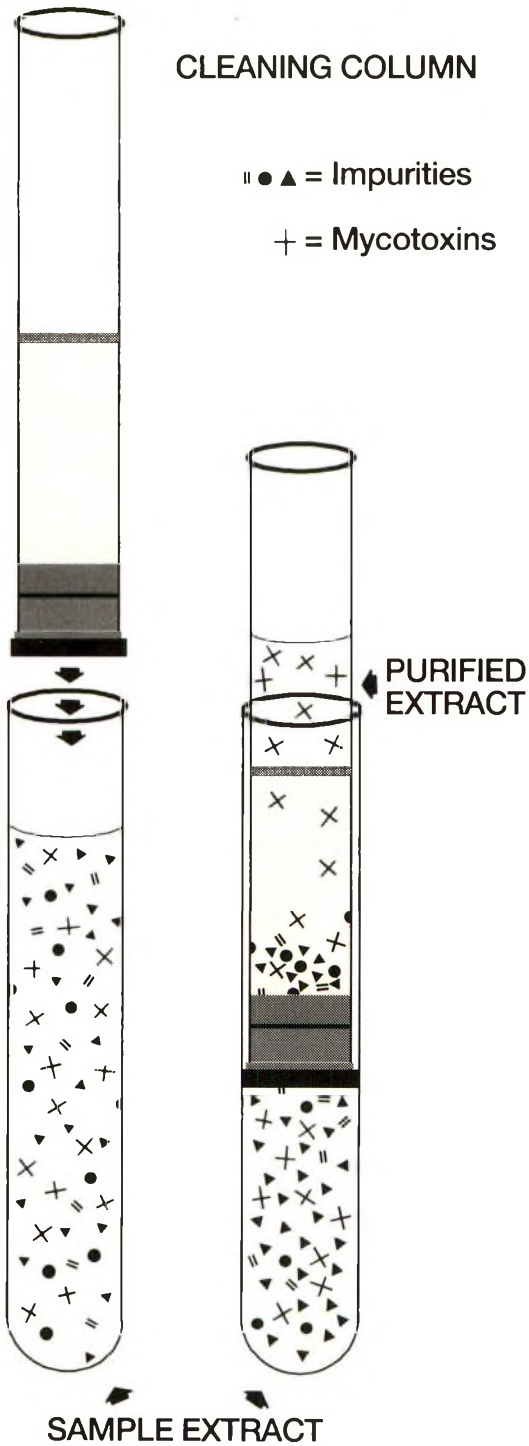

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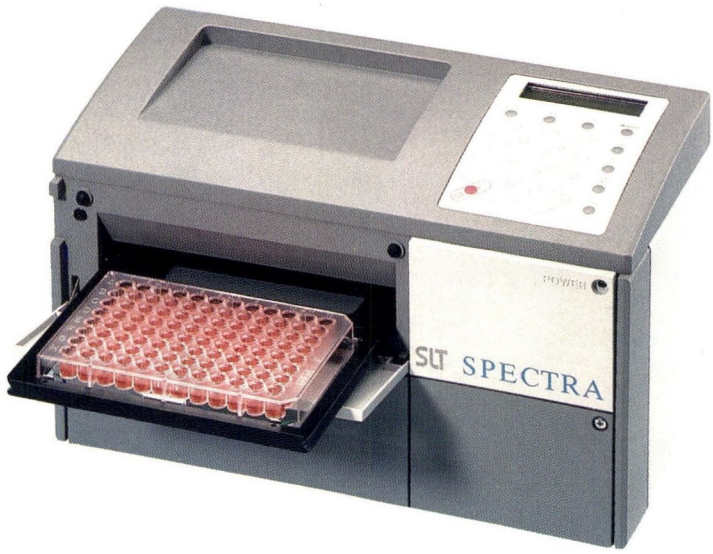

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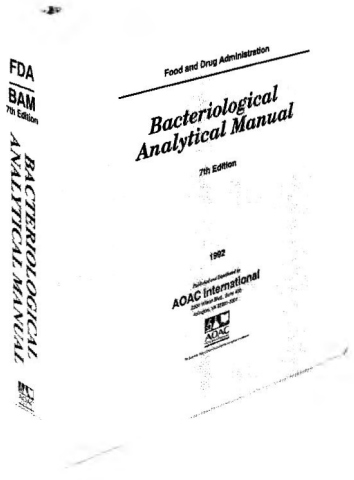

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CONTENTS

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Table of Contents

May/June 1994 VOL. 77, NO. 3 JAINEE 77(3)553-794 (1994)

57A ***Letter from the Editor-in-Chief***
58A ***For Your Information***
68A ***New Products***
72A ***Books in Brief***
75A ***Preliminary Program for the 108th Annual AOAC INTERNATIONAL Meeting and Exposition***
93A ***Cumulative Author Index***

DRUGS, COSMETICS, FORENSIC SCIENCES

553 Determination of Malachite Green Residues in the Eggs, Fry, and Adult Muscle Tissue of Rainbow Trout (*Oncorhynchus mykiss*)
John L. Allen, Jane E. Gofus, and Jeffery R. Meinertz

558 Determination of Sulfamethazine in Bovine and Porcine Tissues by Reversed-Phase Liquid Chromatography
Joe O.K. Boison and Lily J. -Y. Keng

565 Analysis of Penicillin G in Milk by Liquid Chromatography
Joe O.K. Boison, Lily J. -Y. Keng, and James D. MacNeil

570 International Validation Study for the Determination of Chloramphenicol in Bovine Muscle
Robert L. Epstein, Carolyn Henry, Kathleen P. Holland, and John Dreas

577 Determination of Semduramicin Sodium in Poultry Liver by Liquid Chromatography with Vanillin Postcolumn Derivatization
Jon F. Ericson, Albert Calcagni, and Martin J. Lynch

583 Gas Chromatographic Determination of *p*-Chloroaniline in a Chlorhexidine Digluconate-Containing Alcohol Foam Surgical Scrub Product
Walter K. Gavlick and Paula K. Davis

587 Niacin II: Identification of Isonicotinic Acid in Niacin by Liquid Chromatography with Diode Array Detection
Ross D. Kirchhoefer

591 Determination of Nitrofuran Residues in Poultry Muscle Tissues and Eggs by Liquid Chromatography
Luxmy Kumar, John R. Toothill, and Koon-Bay Ho

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596 Gas Chromatographic Determination of Chloramphenicol Residues in Shrimp: Interlaboratory Study
Robert K. Munns, David C. Holland, José E. Roybal, Joseph M. Storey, Austin R. Long, Guy R. Stehly, and Steven M. Plakas

FOOD BIOLOGICAL CONTAMINANTS

602 Deoxyribonucleic Acid Hybridization Method for the Detection of *Listeria* in Dairy Products, Seafoods, and Meats: Collaborative Study
Michael S. Curiale, Terri Sons, Luanne Fanning, Wendy Lepper, Dawn McIver, Susan Garramone, and Mark Mozola

617 Detection of Pork in Heated Meat Products by the Polymerase Chain Reaction
Rolf Meyer, Urs Candrian, and Jürg Lüthy

623 Enumeration of Total Bacteria in Raw and Pasteurized Milk by Reflectance Colorimetry: Collaborative Study
Gary H. Richardson, James T.C. Yuan, Donald V. Sisson, and Barry O. Stokes

FOOD CHEMICAL CONTAMINANTS

628 Determination of Deoxynivalenol in 1991 U.S. Winter and Spring Wheat by High-Performance Thin-Layer Chromatography
Cecilia Fernandez, Michael E. Stack, and Steven M. Musser

631 Detoxification of Citrinin and Ochratoxin A by Hydrogen Peroxide
Sophie G. Fouler, Alka B. Trivedi, and Naofumi Kitabatake

637 Solvent-Efficient Thin-Layer Chromatographic Method for the Determination of Aflatoxins B_1, B_2, G_1, and G_2 in Corn and Peanut Products: Collaborative Study
Douglas L. Park, Mary W. Trucksess, Stanley Nesheim, Michael Stack, and Richard F. Newell

647 Determination of Volatile Organic Contaminants in Bulk Oils (Edible, Injectable, and Other Internal Medicinal) by Purge-and-Trap Gas Chromatography/Mass Spectrometry
Don W. Thompson

655 Enzyme-Linked Immunosorbent Assay of Total Aflatoxins B_1, B_2, and G_1 in Corn: Follow-up Collaborative Study
Mary W. Trucksess and Michael E. Stack

659 Predicting the Distribution of Aflatoxin Test Results from Farmers' Stock Peanuts
Thomas B. Whitaker, Francis G. Giesbrecht, Jeremy Wu, Winston M. Hagler, Jr, and Floyd E. Dowell

FOOD COMPOSITION AND ADDITIVES

667 Determination of Polymerized Triglycerides in Frying Fats and Oils by Gel Permeation Chromatography: Interlaboratory Study
Paul R. Beljaars, Remmelt van Dijk, and Adrie A.M. Houwen-Claassen

672 Determination of *p*-Toluenesulfonamide in Ice Cream by Combination of Continuous Flow and Liquid Chromatography: Summary of Collaborative Study
Paul R. Beljaars, Remmelt van Dijk, and Arie Brands

674 Determination of the Iodine Value of Oils and Fats: Summary of Collaborative Study
David Firestone

677 Gas Chromatographic Determination of Mono- and Diglycerides in Fats and Oils: Summary of Collaborative Study
David Firestone

681 Effect of Various Commercially Available Enzymes in the Liquid Chromatographic Determination with External Standardization of Thiamine and Riboflavin in Foods
Margareta Hägg

687 Determination of Total Dietary Fiber in Foods and Products with Little or No Starch, Nonenzymatic–Gravimetric Method: Collaborative Study
Betty W. Li and Maria S. Cardozo

690 Determination of Soluble Dietary Fiber in Foods and Food Products: Collaborative Study
Leon Prosky, Nils-Georg Asp, Thomas F. Schweizer, Jonathan W. DeVries, Ivan Furda, and Sungsoo C. Lee

695 Determination of Honey Authenticity by Anion-Exchange Liquid Chromatography
Kevin W. Swallow and Nicholas H. Low

703 Enzymatic/Chemical Analysis of Dietary Fiber
Olof Theander, Per Åman, Eric Westerlund, and Hadden Graham

RESIDUES AND TRACE ELEMENTS

710 Liquid Chromatographic Fluorescence Method for the Determination of Thiabendazole Residues in Green Bananas and Banana Pulp
Rene V. Arenas and Nelson A. Johnson

714 Analysis of Heavy Metals in *Aceto Balsamico Tradizionale di Modena* by Flame Atomic Absorption Spectroscopy
Fulvio Corradini, Luigi Marcheselli, Andrea Marchetti, Carlo Preti, and Claudio Biancardi

718 Rapid Abrasion Test To Indicate Lead on the Surface of Ceramicware
Scott P. Dolan

722 Determination of Zinc in Serum, Blood, and Ultrafiltrate Fluid from Patients on Hemofiltration by Graphite Furnace/Atomic Absorption Spectroscopy or Flow Injection Analysis/Atomic Absorption Spectroscopy
Oroncio Jimenez de Blas, Ramon Seisdedos Rodriguez, Jesus Hernandez Mendez, Jose Antonio Sanchez Tomero, B. de Leon Gomez, and Secundino Vicente Gonzalez

728 Analysis of Flusilazole and Its Major Phenyl Metabolite (IN-F7321) by Approaches Based on Gas Chromatography
Richard A. Guinivan and Michael R. Gagnon

735 Preparation of Spiked Soils by Vapor Fortification for Volatile Organic Compounds Analysis
Alan D. Hewitt

738 Packed-Column Gas Chromatographic Method for the Simultaneous Determination of 10 Pyrethroid Insecticide Residues in Fruits, Vegetables, and Grains
Guo-Fang Pang, Chun-Lin Fan, Yan-Zhong Chao, and Tie-Sheng Zhao

748 Spectrophotometric Determination of Decamethrin and Its Residues in Insecticidal Formulations and in Water
R.V. Prabhakara Raju and R. Raghava Naidu

752 Liquid Chromatographic Determination of Norflurazon and Its Initial Metabolite in Soil
William T. Willian and Thomas C. Mueller

TECHNICAL COMMUNICATIONS

756 Quantitative Procedure for Chlorophyllin Copper Complex
Simon Chernomorsky

758 Determination of Reserpine in Tablets by Liquid Chromatography with Fluorescence Detection: Revised Procedure
Ugo R. Cieri

760 Simple and Rapid Determination of Phytase Activity
Adrianus J. Engelen, Fred C. van der Heeft, Peter H.G. Randsdorp, and Ed L.C. Smit

765 Analysis of Streptomycin and Dihydrostreptomycin in Milk by Liquid Chromatography
Geoff C. Gerhardt, Craig D.C. Salisbury, and James D. MacNeil

768 Voltametric Behavior of Zopiclone: Polarographic Determination in Tablets
Juan A. Squella, Juan C. Sturm, Alejandro Alvarez-Lueje, and Luis J. Núñez-Vergara

AOAC SYMPOSIUM: AUTOMATED GOOD LABORATORY PRACTICE

774 Automated Analysis with Computerized Information Transfer in a University Hospital
Claude Bachmann and Olivier Boulat

777 Development of Data Sets for the Validation of Analytical Instrumentation
Stephen L.R. Ellison, Maurice G. Cox, Alastair B. Forbes, Bernard P. Butler, Simon A. Hannaby, Peter M. Harris, and Susan M. Hodson

782 Information Technology/Laboratory Information Management Systems: Problems of Regulatory Compliance
Alan K. Rawling

785 Automation and Quality in Analytical Laboratories
Miguel Valcárcel and Angel Ríos

789 Corruption at the Data Capture Stage and Good Laboratory Practices
Engelbert Ziegler and Horst Lenk

Letter from the Editor-in-Chief

During the past 2 years, research article submissions to the *Journal of AOAC INTERNATIONAL* have doubled. This is due to proactive measures undertaken by AOAC INTERNATIONAL's Editorial Board and staff in 1991 and 1992. The result, however, has been a substantial backlog of accepted manuscripts in 1993 and a publication delay of 10 months after article acceptance. This publication timeline is unacceptable to AOAC, as I'm sure it is to you the authors.

As Editor-in-Chief, I want to inform you of the measures that AOAC is taking to reduce the time from article acceptance to publication, and what steps are being taken to assure such a delay will not happen in the future.

To reduce the backlog, AOAC considered a number of possibilities, among them publishing additional issues. Because additional staff with the necessary experience was not available to produce high quality extra issues, this was not a viable solution. The best and most efficient way to reduce the backlog was to publish more articles per issue. Beginning in 1993, AOAC began publishing bigger issues, with over 40 manuscripts published in each issue, as compared to 28 prior to 1993. In 1994, AOAC will be publishing approximately 45 manuscripts in each issue and expects the backlog to be completely depleted by the end of the year.

I want to assure our authors that the current length of time to publish articles is a temporary circumstance and pledge that, after this backlog is depleted, manuscripts submitted to the *Journal* will average submission to publication times comparable to those of competing journals.

How can we keep this pledge? *Journal* issues will vary in size based on the number of accepted articles awaiting publication. Thus, the more accepted manuscripts awaiting publication, the bigger the *Journal* issues.

Therefore, I ask for your patience and apologize for the delay in the publication of your research. We are very interested in publishing your future research efforts. As a journal, we strive to meet the needs of scientists by publishing informative, quality research articles, collaborative studies, and timely review articles. We could not do this without you and we thank you for your continuing support of the *Journal*.

— Jim Tanner

For Your Information

Meetings

June 2, 1994: AOAC Mid-Canada Section Meeting, Winnipeg, Manitoba, Canada. Contact: Jane Weitzel, Manitoba Department of Energy and Mines, 745 Logan Ave, Winnipeg, MB, R3E 3L5, Canada, telephone +1 (204) 945-2590.

June 13–15, 1994: AOAC Midwest Section Meeting, Columbia, MO. Contact: George Rottinghaus, University of Missouri-Columbia, Veterinary Medical Diagnostic Laboratory, PO Box 6023, Columbia, MO 65211, telephone +1 (314) 882-5994.

June 21–23 1994: AOAC Pacific Northwest Section Meeting, Olympia, WA. Contact: Isabel C. Chamberlain, U.S. Environmental Protection Agency, Region 10, 7411 Beach Dr East, Port Orchard, WA 98366, telephone +1 (206) 871-8706.

September 10–11, 1994: AOAC Short Courses (in conjunction with AOAC Annual Meeting), Portland, OR. Topics: QA for Microbiological Laboratories, Statistics for Methodology Development, Analytical Methods Development, ISO 9000. Contact: AOAC Meetings and Education Department, AOAC INTERNATIONAL, 2200 Wilson Blvd, Suite 400, Arlington, VA 22201-3301, telephone +1 (703) 522-3032.

September 12–15, 1994: The 108th AOAC INTERNATIONAL Annual Meeting and Exposition, Portland, OR. Contact: AOAC Meetings and Education Department, AOAC INTERNATIONAL, 2200 Wilson Blvd, Suite 400, Arlington, VA 22201-3301, telephone +1 (703) 522-3032.

September 15–16, 1994: AOAC Short Courses (in conjunction with AOAC Annual Meeting), Portland, OR. Topics: QA for Analytical Laboratories, How to Testify as an Effective Witness, Good Laboratory Practices. Contact: AOAC Meetings and Education Department, AOAC INTERNATIONAL, 2200 Wilson Blvd, Suite 400, Arlington, VA 22201-3301, telephone +1 (703) 522-3032.

September 29–30, 1994: AOAC Europe Section Meeting, Nyon, Switzerland. Contact: T. Rihs, Swiss Federal Research Station for Animal Production, CH-1725 Posieux, Switzerland, telephone +41 37 877 111.

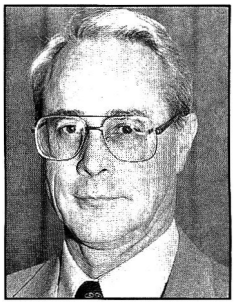
October 25–26, 1994: AOAC Central Europe (proposed subsection) Section Meeting, Smolenice, Slovokia. Contact: Pavel Farkas, Food Research Institute, Priemyselna 4, PO Box 25, Bratislava. Slovokia, telephone +41 7 61355.

December 12–16, 1994: AOAC Short Courses, Baltimore, MD. Topics: Lab Waste Disposal, Environmental Compliance, and Safety; Analytical Methods Development; Good Laboratory Practices; ISO 9000. Contact AOAC Meetings and Education Department, AOAC INTERNATIONAL, 2200 Wilson Blvd, Suite 400, Arlington, VA 22201-3301, telephone +1 (703) 522-3032.

AOAC INTERNATIONAL Names 1994 Harvey W. Wiley Award Winner

William Harvey Newsome, Head of the Pesticide Section, Food Research Division of Health Canada, has been named to receive AOAC's Harvey W. Wiley Award, the most prestigious recognition extended by AOAC INTERNATIONAL for outstanding contribution to methodology. Newsome will receive the award at the opening session of the 108th AOAC INTERNATIONAL Annual Meeting and Exposition in Portland, OR, September 12–15, 1994.

In a career spanning 25 years, all spent with the Health Canada, Newsome has repeatedly demonstrated skill at conceiving analytical methods that have helped further Health Canada's mission of consumer protection. Newsome's methods are used extensively in the control of pesticide residues in Canadian foods.



Within a career marked by accomplishments, 4 are particularly noteworthy for their contribution to the advancement of analytical methodology:(*1*) Newsome developed methods for determining parent EBDC fungicides and 5 of their decomposition products (ETU, ETC, ETMS, EU, and I), and successfully applied these methods in field studies to determine the dynamics of EBDCs and the decomposition/metabolic products of EBDCs in foods. In concomitant studies, Newsome demonstrated for the first time how ETU (a suspected animal carcinogen and teratogen) is formed when foods containing EBDCs are cooked. This discovery resulted in the elimination of many field uses of EBDCs and in the promulgation of a regulation under the Canadian Food and Drugs Act to prohibit the sale of food containing ETU.

(*2*) Newsome also developed methods for the fumigants dibromochloropropane, dichloropropene, and ethylene dibromide, and for the insecticide rotenone and its major metabolite, rotenolone, in foods. Application of these methods in field trials demonstrated for the first time the persistence of dibromocholorpropane and rotenone.

(*3*) The plant growth regulators maleic hydrazide and succinic acid dimethylhydrazide were studied by Newsome because of their potential to degrade to the corresponding free hydrazine. In these investigations, Newsome developed methods for maleic hydraz-

ide, the β-D-glucoside metabolite, and hydrazine. In field studies, he applied the methods both at harvest and storage to show that free hydrazine was not formed as a metabolite. After a GLC method was developed for determining succinic acid dimethylhydrazide, which also permitted the determination of dimethylhydrazine in foods, Newsome demonstrated how UDMH is formed when foods containing the parent hydrazide are cooked. The ramifications of this discovery became most apparent during the recent Alar episode—an episode when Newsome's methods were used extensively to assess the level of contamination of Canadian foods.

(*4*) Newsome demonstrated his organosynthetic skills in the synthesis of several members of 2 classes of compounds (chlorinated diphenyl ethers and chlorinated phenoxy phenols) known to occur in PCP preparations and known to be precursors to chlorinated dibenzofurans. Newsome subsequently developed methods for these compounds in foods and identified chlorinated diphenyl ethers in pork and fish samples for the first time in Canada.

One of Newsome's innovations deserves special emphasis. He recently investigated the use of immunochemistry for the determination of pesticides and environmental contaminants in foods. By synthesizing analogues of the target compounds, deriving immunogens, and raising antibodies (as reagents) in rabbits, he developed immunoassays (RIA and ELISA) for some 9 fungicides and herbicides, which are difficult to determine by conventional methods, and for PCBs in milk and blood. These procedures are more cost-effective and rapid than existing approaches.

Not collaboratively studied, Newsome's methods are instead research methods that detail cause-and-effect relationships. On the other hand, as an AOAC General Referee for Organonitrogen, he has overseen the adoption of 3 methods. He became a member of AOAC in 1971 and first acted as a General Referee in 1975. He has been a General Referee ever since, and also serves on the AOAC Editorial Board. The Association recognized his contributions when last year, he received the honor of being elected a Fellow. Newsome's career in analytical chemistry has been further distinguished by the publication of over 50 scientific papers and reviews and by numerous presentations at professional meetings. Finally, it would be remiss not to mention the multi-disciplinary segment of Newsome's research in which he has made extensive contributions with toxicologists, biochemists, and others in the biological field. Newsome's contributions in these studies include the synthesis of the chemicals to be studied, in addition to the development and application of the analytical techniques required.

Dietary Supplement Regulations—Identifying Analytical Methods for Compliance

Final U.S. Food and Drug Administration (FDA) regulations for nutritional labeling of dietary supplements were published in the *Federal Register* (1) on January 4, 1994, with full compliance being required by July 1, 1995. This final rule basically treats dietary supplements as foods. However, for supplements of any vitamin or mineral with an established Reference Daily Intake (RDI) value, the new rule requires labels that are similar yet different from those required for conventional foods. All other types of dietary supplements (e.g., herbs, amino acids, and other nutritional substances) must use the same nutrition labeling format

as conventional foods, which must comply with the 1993 foods nutritional labeling regulations (2) by May 8, 1994, (or July 6, 1994, if regulated by the U.S. Department of Agriculture).

The 1993 foods regulations specify mandatory labeling for 14 proximates, vitamins, and minerals, and voluntary labeling for 23 other parameters (listed in Table 1). Likewise, the regulations for dietary supplements specify that the same parameters be presented on their nutritional labels, plus chloride, chromium, fluoride, manganese, molybdenum, selenium, and vitamin K. These additional 7 nutrients have also been proposed (3) for the conventional foods regulations.

Although FDA treats dietary supplements as foods in the dietary supplement final rule, the analytical methods that are being used to analyze conventional foods for nutritional labeling purposes normally would not be applicable for analysis of most dietary supplements (especially those in tablet or capsule form) for nutritional labeling purposes. Based on the "serving size" of most dietary supplements in tablet or capsule form, they will have insignificant amounts of the macronutrients, such as protein, carbohydrates, and fat. In these cases, dietary supplement regulations will not require that such products be analyzed for the macronutrients present in insignificant amounts, nor have them declared on the labels.

The U.S. Pharmacopeial Convention's (USP) *Pharmacopeial Forum* notes that "nutritional supplements" are also often called "dietary supplements," implying that the 2 terms are essentially synonymous. In a 1993 speech, FDA Deputy Commissioner for Policy Michael Taylor defined "nutritional supplements" as products consisting solely of vitamins and essential minerals (4). As such, they are a subset of the larger world of "dietary supplements," which also includes a large number of other products, such as herbs, and high-potency amino acid supplements. According to Taylor, nutritional supplements account for over 80% of the multi-billion dollar dietary supplement market.

Table 1. Nutrients for nutritional labeling of dietary supplements of vitamins and minerals

Nutrient	Mandatory/ Voluntary[1]	RDI or DRV[2]	Units	Nutrient	Mandatory/ Voluntary[1]	RDI or DRV[2]	Units
Calories	Mandatory	2000	Cal	Riboflavin	Voluntary	1.7	mg
Calories from fat	Mandatory		Cal	Niacin	Voluntary	20	mg
Calories from saturated fat	Mandatory		Cal	Vitamin B_6	Voluntary	2	mg
Fat, total	Mandatory	65	g	Folate	Voluntary	0.4	mg
Saturated fat	Mandatory	20	g	Vitamin B_{12}	Voluntary	6	µg
Polyunsaturated fat	Voluntary		g	Biotin	Voluntary	0.3	mg
Monounsaturated fat	Voluntary		g	Pantothenic Acid	Voluntary	10	mg
Cholesterol	Mandatory	300	mg	Calcium	Mandatory	1	g
Carbohydrate, total	Mandatory	300	g	Iron	Mandatory	18	mg
Dietary fiber	Mandatory	25	g	Phosphorus	Voluntary	1	g
Soluble fiber	Voluntary		g	Iodine	Voluntary	150	µg
Insoluble fiber	Voluntary		g	Magnesium	Voluntary	400	mg
Sugars	Voluntary		g	Zinc	Voluntary	15	mg
Other carbohydrate	Voluntary		g	Selenium[3]		70	µg
Protein	Mandatory	50	g	Copper	Voluntary	2	mg
Vitamin A	Mandatory	5000	IU	Manganese[3]		3.5	mg
% Vit. A as beta-carotene	Voluntray			Fluoride[3]		3	mg
Vitamin C	Mandatory	60	mg	Chromium[3]		130	µg
Vitamin D	Voluntary	400	IU	Molybdenum[3]		160	µg
Vitamin E	Voluntary	30	IU	Chloride[3]		3400	mg
Vitamin K[3]		80	µg	Sodium	Mandatory	2400	mg
Thiamin	Voluntary	1.5	mg	Potassium	Voluntary	3500	mg

[1] Mandatory if fortified or health claims made about nutrient.
[2] RDI = Reference Daily Intake; DRV = Daily Reference Value.
[3] Additional nutrients required (if present) for dietary supplements. RDIs not established, indicated levels proposed 1/4/94.

Method Requirements

The dietary supplement final rule specifies the analytical methodology to be used for compliance purposes only in general terms. In the preamble (comments) section of the rule, FDA indicates that it will analyze samples using "*Official Methods of Analysis of AOAC INTERNATIONAL*, 15 edition, or if no AOAC method is available or appropriate, by other reliable and appropriate analytical procedures. Manufacturers, however, are free to use whatever methodology they believe will give results consistent with methods used by FDA...."

Only a few AOAC Official Methods are available for sample types resembling nutritional supplements in tablet and capsule form, and these only partially cover the list of required nutrients. They include one or more methods for vitamins C, D, and E, thiamin, riboflavin, niacin, vitamin B_6, folic acid, vitamin B_{12}, and pantothenic acid in at least one of the following matrix types: a premix, concentrate, drug, or vitamin/multivitamin "preparation." Manufacturers technically may be free to use whatever methods they believe will give results consistent with those the FDA will use for the full range of nutrients. How can manufacturers, however, verify that the methods they use for quality control and release testing of specific products will give results that are consistent with those the regulators may choose for compliance purposes?

By contrast, several hundred AOAC® Official Methods are available for conventional foods analyses (5), including suitable methods for nutritional labeling analyses (6, 7). These methods can no doubt be applied to some dietary supplements, but only after appropriate modification and revalidation of the methods so modified. All of these methods have successfully passed the rigorous AOAC collaborative study process on one or more foods. In addition, they were extensively and systematically reviewed and evaluated for nearly 1½ years (from December 1991 to April 1993) by the AOAC Task Force on Methods for Nutritional Labeling Analyses (of which the author was a member) for their applicability to conventional foods, but not for dietary supplements. The Task Force was composed of over 40 chemists, food scientists, and other interested professionals representing government, industry, academia, and independent laboratories. The work of the Task Force, as documented in the book *Methods of Analysis for Nutritional Labeling* (7), is expected to benefit the food industry.

Meanwhile, the USP Advisory Panel on Analytical Methods for Multivitamin and Mineral Combination Products and Enteral Products has worked extensively since December 1990 with the USP Subcommittee on Vitamins, Minerals, and Enterals (8) to review, revise, and adopt a comprehensive set of analytical methods for 6 classes of nutritional supplements. Most of these methods have been carried through the entire USP revision process for USP-NF standards before being issued as final methods in the USP XXII supplements (9–11). Many companies perceive a marketing advantage from using USP methods so they can indicate on their label that the product conforms to USP specifications.

In the area of quality control and quality assurance for the manufacture of dietary supplements, the Council for Responsible Nutrition (CRN) published a comprehensive "Nutritional Supplement Good Manufacturing Practice Guidelines" (12), which had been adopted by its member companies. Many of the guidelines were derived from the Current Good Manufacturing Practice (CGMP) regulations for drugs.

The USP Subcommittee on Vitamins, Minerals, and Enterals later modified the CRN guidelines slightly so that they more closely follow CGMPs, and adopted them (13) as USP "Manufacturing Practices for Nutritional Supplements." The CRN guidelines and the USP manufacturing practices stipulate that the methods used for analysis need to be specified in writing, but do not stipulate that they be validated and documented to the same degree, as do CGMPs. Only CGMPs require a stability testing program to establish expiration dates.

As an independent contract laboratory, Southern Testing and Research Laboratories, Inc., has analyzed a large number of nutritional (dietary) supplements from multiple manufacturers, using the final USP methods for nutritional supplements published in USP XXII supplements 7, 8, and 9. Working within the method adjustments allowed by USP, we found that most of the methods performed adequately on a wide variety of multivitamin and multimineral sample types analyzed. In cases where the methods did not perform well, it was primarily due to the methods not being optimized for the matrix analyzed. Most of these cases involved insufficient flexibility in the method to allow for the wide range in concentration of active ingredients encountered, interferences between some active ingredients, or interferences between active ingredients and some excipients.

USP policy allows use of only those inactive ingredients (excipients) in a formulation that do not interfere with the USP methods being used to determine the level of any active ingredient(s) present. While this may be reasonable to require in formulations with only a few active ingredients, it is not practical for multivitamin/multimineral formulations that may have as many as 15 or more active ingredients.

Currently, about the only alternative for manufacturers, if any excipient interferes with the analysis by the USP method of an active ingredient, is to reformulate the product. In a few cases, an alternate USP method of analysis may be available. Needless to say, this is a very costly approach to solving the analytical problem, especially if the product is already on the market. Another approach would be to write enough flexibility into the methods to allow the analyst to eliminate minor interferences, provided modifications are validated by the analyzing laboratory, following standard USP validation procedures.

Other comments we received regarding USP methods addressed improving speed and efficiency, reducing quantities of reagents required, and reducing amount of waste generated. One suggestion for making the methods more efficient allows the use of either multianalyte or single analyte technologies, or procedures, where the 2 are shown to give equivalent results. Examples would be the simultaneous determination of multiple minerals by inductively coupled plasma emission spectroscopy vs determination of one mineral at a time by atomic absorption spectroscopy; or using one extraction for determination of several vitamins, and so forth.

Need for AOAC Validation

In order for AOAC to provide methods that will serve the multiple needs of efficiency, quality control, compendial conformance, and regulatory compliance for the full range of dietary supplement types expected to need analyses, we believe that methods from multiple sources, including those of AOAC, USP, and the regulatory industry, should be considered. How can this be achieved?

First, all interested parties should weigh the positive benefits of AOAC recognition of existing USP nutritional supplement methods vs the additional costs and any disadvantages AOAC recognition might bring. Obviously, FDA, USP, and the regulated industry would each have to confirm that a net positive benefit exists before proceeding to the next step.

Second, representatives of the dietary supplement industry, FDA, USP, the independent laboratory community, and other interested parties could meet, much as we did for conventional foods, to discuss both the strengths and deficiencies in the currently available methods, identify those methods that are satisfactory by independent verification for dietary (including nutritional) supplement analyses, identify areas where further work needs to be done to produce satisfactory methods, and begin the process of validation for AOAC acceptance.

Third, AOAC has recently established a new category of validated methods, AOAC Peer-Verified Methods (14). These are methods that have been found to be satisfactory in at least 2 laboratories, according to performance testing criteria established by AOAC and after AOAC expert review. This program could be a means to quickly gain a level of AOAC recognition that may be suitable for compliance purposes. At the same time, manufacturers could use the same methods for product quality control and release, and for certifying compliance with compendium requirements on their product labels.

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—Kenneth Boyer
Southern Testing and Research Laboratories, Inc.

Meet Us in Portland

The 108th AOAC INTERNATIONAL Annual Meeting and Exposition will be held September 12–15, 1994, at the Red Lion Hotel in Portland, Oregon. The scientific program promises to be innovative and interesting.

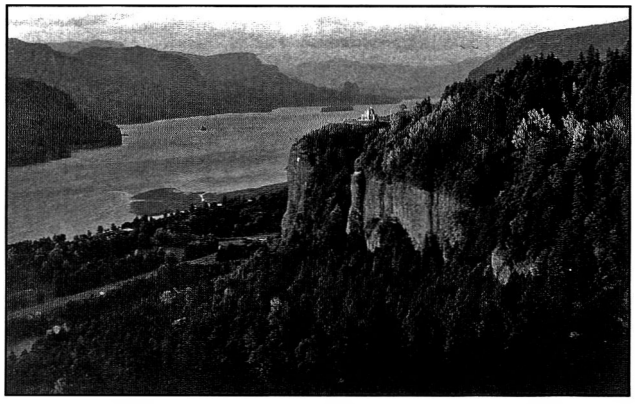
So does Portland, Oregon.

This is to entice analytical chemists to meet each other in Portland. Details of the program are published in this issue of the Journal of AOAC INTERNATIONAL.

A travel guide proclaims, "In Portland, 20-pound salmon are as common as 20-story office buildings." From the MAX Light Rail System to parks and recreation areas, Portland indeed displays incredible diversity in a compact and friendly atmosphere. One of the greatest things about visiting Portland is also discovering what is beyond Portland: the Columbia River and Mt. St. Helens to the north; Mt. Hood and the Cascade range to the east; wine country to the south; and the Pacific Coastline to the west. So, when you come to Portland for the AOAC annual meeting, as the travel guide declares, "Don't rule out anything."

Urban Excursions

Just contemplating the assortment of outdoor activities within the Portland metro area may exhaust the imagination, if not the body. Pleasure boating on the Willamette or Columbia ranges from sternwheelers to kayaks. Sports fishing on the Willamette, Clackamas, and Columbia rivers yield Chinook salmon, steelhead trout, and sturgeon. Nearby lakes are stocked with bass and trout. Bird watchers at Oaks Bottom or Sauvie Island can sight bald eagles, blue herons, and sandhill cranes.



An easy day trip for AOAC annual meeting attendees—the Columbia River Scenic Route north of Portland takes you to Crown Point, a favorite vantage point of the Columbia Gorge for both sightseers and photographers

Hiking through city parks takes you past old-growth groves, as well as open vistas that extend 100 miles to Washington's Mt. Rainier. For golfers, there are more than 35 public and 15 private courses. Horseback riders can ride through grassland preserves at Powell Butte or visit nearby ranches.

If your idea of a workout is a leisurely stroll, discover any number of outdoor spaces or colorful neighborhoods. One attraction is Leach Botanical Gardens. Established on the grounds of a local botanist's home, these public gardens now display 1,500 species of native plants.

On the other side of town, natural and man-made beauty complement each other at the site of the Pittock Mansion. Whether touring this 1914 estate, or picnicking on its lawns, a panoramic view takes in the valley and mountains.

In Washington Park, the Japanese Gardens have been cited as the most authentic outside of Japan. The International Rose Test Garden delights visitors with 450 blooming varieties of Portland's signature flower. Hop on the Washington Park Zoo railway and see the nation's largest elephant herd. The zoo operates the world's most successful breeding program for these endangered animals. Even a century ago, Portland's zoo was called "A model for all zoos for the confinement of wild animals." Today visitors can learn about 192 species and experience special exhibits and natural habitats, such as the Alaska Tundra and African Rain Forest.

Indoor Adventures

The Oregon Historical Society recognized early settlers' historical signifi-

cance and began in 1873 as the Oregon Pioneer Association, with membership limited to persons who had migrated before 1853. However, they soon realized it would face a dwindling membership, so they changed their name and mission. Their recently renovated History Center encompasses an entire city block. Among its artifacts are a 12,000-year-old sagebrush sandal and more than 3 million photographs.

The World Forestry Center began as "The Wood Parthenon," a giant log cabin built for the Lewis and Clark exposition in 1905. Today, its campus has several exhibit halls. Features include a 70-foot "talking" Douglas fir, a forest fire simulation, and the Jesup Collection, which displays every tree species indigenous to North America. The Oregon Museum of Science & Industry (OMSI) outgrew its Washington Park location. Its new $40 million complex on the east bank of the Willamette ranks among the top 10 science centers in the United States. Visitors experience exhibits and attractions such as the simulated earthquake room, a 4-story OMNIMAX screen (featuring the Mt. St. Helens eruption), a tornado-in-a-box, and a planetarium. A new museum represents one of the American West's oldest heroes. "Cowboys Then and Now" traces the evolution of the cowboy, and sets us straight on Hollywood myth. At the authentic chuck wagon, a "talking head" named Zack tells what it was really like to eat dust on the cattle drive.

For those who are nostalgic about highway drives, the complete set of "Burma Shave" signs can be seen at the American Advertising Museum in Portland's Old Town. This one-of-a-kind museum features memorabilia ranging form 4 centuries of print ads to an outdoor neon gallery.

Day Trips

Although there is much to discover within the city limits, day trips to the nearby mountains open whole new worlds. It is a 56-mile drive to Mt. Hood, Oregon's highest peak. This 11,235-foot summit is so famed for its year-round skiing that Olympic teams train here in the summer; hikers wind their way toward waterfalls. Historic Timberline Lodge has all the views of the mountain and valley below and is an example of craftsmanship of the 1930s Works Progress Administration (WPA).

Dotted with cascades and wilderness terrain, the Columbia River Gorge invoked as much terror as wonder among early pioneers. Places with names like Starvation Creek remind us that in 1884, natural forces could delay travel for 3 weeks. Today's visitors can reach this National Scenic Area in less than one-half hour. Modern thrill-seekers now windsurf, or hikers climb Mt. Defiance. There are plenty of "soft adventures" in the Columbia Gorge, too. Along this route is the Hood River Valley, famous for its pear and apple orchards; liqueurs are distilled from the fruit. Flanked by Mt. Hood and Washington's Mt. Adams, the views are distinctly Oregon.

West of Portland are the vineyards in the Willamette and Tualatin valleys. Oregon wines are attracting international attention as well as foreign investment. You don't have to be a wine connoisseur to savor the drive through these rolling hills.

Farther west is Oregon's coastline, where a newly opened aquarium is nestled between bluffs and seashore. The state's landmark land-use laws mean public access to beaches all along the coast, making it a kiter's and walker's heaven.

Meet us in Portland for the 108th AOAC INTERNATIONAL Annual Meeting—you'll enjoy your visit.

AOAC Thanks Contributors to Harvey W. Wiley Awards Fund

The Association thanks the members of AOAC INTERNATIONAL listed below for their contributions to the Harvey W. Wiley Awards Fund. This fund supports both the Harvey W. Wiley Award, which rewards scientists for outstanding contributors to analytical methodology, and the Harvey W. Wiley Scholarship Award, which provides support to deserving students majoring in fields relevant to the mission of AOAC INTERNATIONAL.

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New Products

EcoCART 125-3 Glass LC Cartridge Columns Improve Resolution and Sensitivity

The new EcoCART LC cartridge column significantly improves resolution and detection sensitivity, and reduces solvent consumption. Designed to provide better resolution with no need to further optimize a conventional LC system, EcoCART 125-3 consists of a unique combination of a pressure resistant glass column with PEEK end fittings housed in a stainless steel cartridge holder with 2 view ports. A system of manu-CART guard column adapters and cap nuts permit easy configuration and assembly. The extremely smooth transparent glass surface is ideal for LC, and permits the viewing of separations of colored substances as they occur. This highly efficient system can cut solvent consumption up to 50%, making it economical as well as easy to use. EM Separations.
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Technical Furniture Line: Addendum Modular Laboratory Systems

Addendum is a modular reconfigurable laboratory system that provides maximum flexibility in constructing complete laboratory environments. Unlike traditional laboratory design, which is difficult to adapt for technological advances, Addendum systems have been designed to support the latest technology and can be easily reconfigured to meet changing needs. Components are completely modular and interchangeable. Service line placement is completely contained within the support frame to provide easy access for maintenance or cleaning without time consuming work disruption. To design a customized laboratory environment, the client may choose from basic support structures of various sizes; then add walls, work benches, cabinets and pedestals, storage units, fume hoods, as well as appropriate electrical, gas, communications and plumbing service panels in virtually any combination. Wright Line.
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m-ColiBlue24™ Broth: Enumerate Total Coliforms and *E. coli* Simultaneously

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Standards for U.S. EPA Method 509 Available for EM Science

New standards of U.S. EPA Method 509 include all required components: a performance check solution, ethylene thiourea standard, internal standard, surrogate standard, and a radical scavenger solution. EM Science.
Circle No. 325 on reader service card.

Channeltron 5778 Mass Spec Detector Meets High Sensitivity Demands

A new Channeltron mass spec detector for the high sensitivity demands of the Hewlett-Packard 5971A and 5972A MSDs is now available from Galileo Electro-Optics Corp. Optimized through computer modeling simulation for maximum signal collection, this easy-to-install detector is ideal for applications where high sensitivity is critical to an instrument's performance. The Channeltron 5778 mass spec detector achieves exceptional sensitivity performance levels. Manufactured for reliability and long life, the detector offers a combination of advanced engineering features that allow analyses of complex mixtures at subpicogram and subfemtogram levels. Key features include ultralow microphonics, optimized ion optics, and photon absorbing hardware. The detector's extended linear dynamic range, a product of Galileo's dual-stage design, results in reproducible spectra and accurate quantification for both trace and high-level analysis, all with an abso-

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lutely linear response exceeding the instruments's limits and all other available detectors. The 5778's long life assures consistently accurate results requiring less frequent calibration and less down time, making it one of the most economical detector's available for the H-P MSDs. The Channeltron 5778 mass spec detector comes in Galileo's patented plug-in, board-mounted design for easy installation. Its new ceramic platform reduces background noise to extremely low levels while improving overall microphonics. To assure accurate and permanent alignment, Galileo sets the detector's critical ion optics at the factory. Galileo Electro-Optics Corp. Circle No. 326 on reader service card.

EM Science Introduces Updated Explosives Mixtures for U.S. EPA Method 8330

An updated explosives mixture for U.S. EPA Method 8330, complete with TNT metabolites, contains 2-amino-4,6-dinitrotoluene and 4-amino-2,6-dinitrotoluene. The explosives are available in individual solutions, in an individual solution set, and in a mixture. EM Science. Circle No. 327 on reader service card.

ABC Instruments Announces Automated Solids Extractor

The Soxtherm Solids Extractor is a highly automated Soxhlet extraction instrument capable of processing 6 samples simultaneously in less than 3 h, and is up to 5 times faster than traditional Soxhlet extraction. Programmed parameters control each of several timed steps of extraction for a completely automated system, and it pays for itself with savings in time, labor, and solvent.

Soxtherm was designed for the high volume demands most laboratories face today: to increase sample throughput while enhancing the extraction process with fully automated, reliable results. It performs U.S. EPA SW-846, Automated Soxhlet Extraction Method 3541, and is suitable for all accepted Soxhlet methods for a wide variety of materials and environmental samples. ABC Instruments. Circle No. 328 on reader service card.

CAPCELL Pak: Novel Packed Column for Liquid Chromatography

CAPCELL Pak is a novel packed column for liquid chromatography, which employs "capsule-type" (polymer) coated silica-based packing that maintains excellent separation and peak sharpness even in alkaline conditions. The packing material is porous, spherical silica gel and its surface is coated with a thin film of silicone polymer; then, an octadecyl group (C_{18}) or other groups are bonded to the polymer. The columns are available in 6 bonded phases of C_{18}, C_8, C_1, phenyl, cyano, and amino; silica pores sizes of 120 and 300Å; and in packed analytical column sizes of 1, 1.5, 2, and 4.6 mm id, with lengths of 15 and 25 cm. The semi-preparative columns are available in 10, 20, and 30 mm id. Also available are preparative columns of 50 and 100 mm id with lengths up to 100 cm. DyChrom. Circle No. 329 on reader service card.

Software for Spectral Processing Introduced

New Spectacle software adds several key features to the strong feature set of Thermo Separation's PC1000 package. Automatic peak purity reporting is new. A peak purity profile graphically displays the purity value across the entire peak. This allows a user to see immediately if the peak is pure or where an impurity is eluting. A peak purity report shows the calculated peak purity value. Spectacle features a toolbar that provides easy, convenient access to the key qualitative controls of isograms, 3-D plots, ratiograms, and spectral comparisons. Also new is automatic library searching. The library searching permits unattended identification of compounds like PAH's in the air and in crude oils, pesticides in drinking water, antibiotic drugs in food, drug metabolites, and naturally occurring pharmaceutically active compounds. PC1000 software with Spectacle now also supports constaMETRIC® and SpectraSYSTEM® pumps, SpectraSYSTEM® detectors, the spectroMONITOR® 5000 photodiode array detector, and the SpectraFOCUS® multi-wavelength, scanning detector. Thermo Separation Products is a respected international manufacturer and supplier of high-quality instruments and systems for liquid chromatography, chromatography data systems, automated mercury analysis and capillary electrophoresis. Thermo Separation Products. Circle No. 330 on reader service card.

Automatic Faucet Controller

This new water controller promises to help eliminate cross contamination at the laboratory sink. It is the first and only battery-powered controller available of its kind. The unit has an ultrasonic sensor that detects motion and automatically turns the water on and off. Now laboratory personnel do not have to touch contaminated faucet handles or worry about the spread of infectious materials. It saves water, too, which is especially useful for expensive high purity water outlets. The controller installs quickly and easily on most existing faucets, costs less, and out performs all competitive infrared devices. It requires non of the expense and inconvenience of electrical wiring and plumbing. A built in microprocessor enables the 9-volt battery to last as long as 1 year. Thomas Scientific. Circle No. 331 on reader service card.

Books in Brief

A Practical Guide to Instrumental Analysis. By Erno Pungor. Published by CRC Press, Inc., 2000 Corporate Blvd, NW, Boca Raton, FL 33431-9868, 1994. 336 pp. Price: U.S. $59.95/Outside U.S. $72.00. ISBN 0-8493-8681-0.

This book covers basic methods of instrumental analysis, including electroanalytical techniques, optical techniques, atomic spectroscopy, X-ray diffraction, thermoanalytical techniques, separation techniques, and flow analytical techniques. Each chapter provides a brief theoretical introduction followed by basic and special application experiments. This book is ideal for readers who need a knowledge of special techniques to use instrumental methods to conduct their own analytical tasks.

A Practical Guide to Environmental Laboratory Services, 1994 Edition. Published by Aquarion Co., 836 Main St, Bridgeport, CT 06601-2353, 1994. 207 pp. Copies may be ordered by contacting IEA's corporate headquarters in Cary, NC, at +1 (919) 677-0090.

Sensing a need in the marketplace to more effectively educate users of environmental laboratory services, IEA, Inc., an environmental testing laboratory subsidiary of Aquarion Co., published this guide to laboratory services. The result of a year-long effort, this comprehensive guide is designed for use by project managers and engineers to facilitate their decision making regarding environmental laboratory services. "This innovative compilation describes in detail every facet of laboratory testing, while providing a balanced and unique view of this vital sector of the environmental services industry," said Richard K. Schmidt, president of IEA. The 207-page guide includes a broad range of topics, such as choosing a laboratory, legal issues surrounding laboratory analysis, an introduction to laboratory services, and a comprehensive discussion of various regulatory applications. Also included are sections devoted to laboratory reports and how to interpret them, and commonly asked questions and answers. Accompanying the text are 16 fully detailed tables, along with appendices that list such useful information as a glossary of environmental laboratory terms and a complete listing of telephone numbers for sate and federal regulatory authorities.

Time-of-Flight Mass Spectrometry. Edited by Robert J. Cotter. Published by the American Chemical Society, 1155 16th St, NW, Washington, DC 20036, 1994. 549 pp. Price: U.S. and Export: $59.95. ISBN 0-8412-2771-3.

This book presents a comprehensive discussion of time-of-flight mass spectrometry. It examines the use of time-of-flight mass spectrometers as tandem instruments and explores interfacing time-of-flight mass spectrometers with other continuous ionization techniques. An overview chapter, a chapter describing the history of time-of-flight mass spectrometry, and 2 chapters describing techniques and examples of applications to biological research are also included.

The Laboratory Environment. Edited by Rupert Purchase. Published by CRC Press, Inc., 2000 Corporate Blvd, NW, Boca Raton, FL 33431-9868, 1994. 270 pp. Price: $85.00. ISBN 0-85186-050-9.

The creation of a safe laboratory environment is dictated not only by basic health and good laboratory practice considerations, but also by the desire to attract and retain talented people in scientific work. This book brings together specialists in architecture, occupational medicine, radiological protection, toxicology, microbiology, health and safety, and chemistry to describe the design and management of the laboratory environment for the 1990s. Essential topics covered include health surveillance of laboratory staff; the classification, handling, and disposal of carcinogens; the recycling of laboratory waste; and the relationship between client and contractor in the design and construction of laboratory areas.

Air Monitoring by Spectroscopic Techniques. Edited by Markus W. Sigrist. Published by John Wiley & Sons, Inc., 1 Wiley Dr, Somerset, NJ 08874-1272, 1994. 531 pp. Price: $85.00. ISBN 0471558753.

Air Monitoring by Spectroscopic Techniques offers scientists and engineers unique sate-of-the-art information on the latest, most promising laser and nonlaser based spectroscopic method used in the detection and analysis of air pollution and trace gases—data previously available only in scattered resources. The opening chapter on the problem of air pollution and monitoring includes comparisons of the various conventional methods now used for trace gas detection and analysis. In the next 5 chapters, leading experts in the field examine the basic characteristics, applications, and advantages and limitations of various spectroscopic, air-monitoring techniques, as well as the instrumentation involved in using each method and its future prospects. Numerous application examples further enable practitioners to compare the various techniques and help show them how and when to apply such methods as differential optical absorption spectroscopy, light detection and ranging, photoacoustic spectroscopy, tunable diode laser spectroscopy, and Fourier transform infrared spectroscopy.

Arsenic in the Environment, Part I: Cycling and Characterization. Edited by Jerome O. Nriagu. Published by John Wiley & Sons, Inc., 1 Wiley Dr, Somerset, NJ 08874-1272, 1994. 430 pp. Price: $95.00. ISBN 0471579297.

The first truly comprehensive and up-to-date investigation of the substance, *Arsenic in the Environment* is a comprehensive 2-volume work that sheds light on arsenic's history, chemical composition, characteristics, behavior in the environment, and the analytical techniques used to measure it (Part I) as well as its impact on human health and the ecosystem (Part II). Each self-contained volume is detailed, well-organized, and offers a wide range of professionals—from chemicals and environmental engineers to toxicologists and industrial hygienists—immediate access to subjects of special interest.

Automatic Atomic-Emission-Spectroscopy. By Karl Slickers. Published by Spectro Analytical Instruments, 160 Authority Dr, Fitchburg, MA 01420, 1994. 500 pp. Price: $63.00. Copies may be ordered by contacting Spectro Analytical Instruments at +1 (508) 342-3400.

This 500 page book, including many drawings and tables, is specifically written for those people who choose to use spectrochemical analysis as a routine application. It provides detailed information on various techniques for the analysis of liquids, powders, and solid samples for environmental protection, process, and quality control. As the author puts it: "Yes, I am certain that computer and fully automated analysis systems that can be integrated like a 'Coca-Cola' vending machines are the future in this field, but we must not forget the basic functions, theories, and techniques necessary to achieve improved accuracy and performance."

ADVANCE REGISTRATION FORM

Registration form for the 108th AOAC INTERNATIONAL ANNUAL MEETING & EXPOSITION

AOAC INTERNATIONAL
The Scientific Association Dedicated to Analytical Excellence

Return to: AOAC INTERNATIONAL, 1970 Chain Bridge Road, Dept. 0742, McLean, VA 22109-0742

Early-Bird Registration Deadline:
postmarked by July 15, 1994

Advanced Registration Deadline:
postmarked by August 25, 1994

Yes! I want to be there for the 108th AOAC INTERNATIONAL Annual Meeting & Exposition, September 12–15, 1994 at the Red Lion Inn Lloyd Center, Portland, Oregon, USA

Name _____

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❒ Yes ❒ No Is this your first AOAC meeting?

❒ Yes ❒ No Will you attend the Business Meeting/Luncheon on Thursday, September 15, 1994.

Name of Guest/Spouse _____

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Special Needs — If you need special accomodations such as an accessible room, please contact the hotel directly. For any other special services required to facilitate your participation in the annual meeting, please contact the AOAC INTERNATIONAL Meetings and Education Department at +1-703-522-3032.

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I. Meeting Registration Fees

Full Meeting	AOAC Members	Non-members*
Early Bird	( ) $225	( ) $290
Advance	( ) $260	( ) $325
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Student	( ) $50	( ) $50

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Exhibit Only	( ) $15	( ) $15
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Workshops:	Member Rate	Non-member Rate
Supercritical Fluid Extraction Applications in Analytical Methodology	$150.00	$175.00
Workshop on Juice Analysis	$275.00	$315.00
Special Session:		
Forum on Methods for Antibiotics and Drugs in Feeds		
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Social Event	Per Person	Amount Enclosed
Tuesday Night Event	❒ $40	$ _____

Total Fee Enclosed for Social Event $ _____(570)

Subtotal I: Meeting Registration + Workshop + Social Event $ _____

II. Short Course Fees

AOAC Courses	AOAC Members	Non-members
One Course	❒ $495	❒ $560
Two or more, each	❒ $445	❒ $510
Improving Your Scientific Writing Skills (Special fee available to members only).	❒ $350	❒ $560

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QA for Analytical Laboratories	September 15-16 $_____	(530)
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How to Testify as an Effective Witness	September 15-16 $_____	(534)

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Program Preview

108TH AOAC INTERNATIONAL
ANNUAL MEETING & EXPOSITION

For Analytical Science That Solves Today's Problems and Anticipates Tomorrow's Needs

Portland, Oregon ◆ Red Lion Inn, Lloyd Center ◆ September 12-15, 1994

Meet the AOAC INTERNATIONAL Meeting Attendee...

The AOAC meeting attendee is someone much like yourself — an analytical scientist or scientist/administrator, chemist, microbiologist or other biologist, or biochemist... working in one or more areas of analysis such as foods or beverages, feeds, fertilizers, pesticides, soil or water, pharmaceuticals, human matrices, hazardous wastes, forensics, or other related materials.

And attendees come from all over the world. They come because they are interested in staying on top of the constant changes within analytical science, to improve their effectiveness on the job, and to meet with other scientists to exchange ideas, discuss problems, and find solutions.

Special Airfares for Registrants

Delta is the official airline for this meeting. For reservations and information on special fares, call Delta Airlines at +1-800-241-6760 and refer to File No. R0133.

Accommodations

The meeting headquarters is the Red Lion Inn Lloyd Center in downtown Portland. The special room rate for AOAC attendees is $99.00 plus tax per night, single and double occupancy. Lodgings are not included in the meeting registration fee. Make arrangements directly with the hotel at +1-503-281-6111 (fax +1-503-284-8553). To receive the discounted rate, mention AOAC INTERNATIONAL.

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Note: Please do not mail, fax or phone in your registration after August 25, 1994. After that date, please plan to register on-site in Portland, Oregon.

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I would like to become an AOAC INTERNATIONAL member and take advantage of the member discount. I am enclosing the $65 AOAC INTERNATIONAL membership fee along with the meeting fees and meeting registration form.

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I hereby apply for membership in AOAC INTERNATIONAL, and if accepted, agree to abide by its rules and bylaws.

Total: $_____ _____

Signature Date

Information as of May 1, 1994. Additional presentations will be added to subsequent program.

Sunday • September 11, 1994

WORKSHOP

Supercritical Fluid Extraction Applications in Analytical Methodology

Chairman: Elizabeth Calvey, Food and Drug Administration

Specific experiments will be identified at a later date. Lectures will deal with fundamentals of supercritical fluids; supercritical fluid extraction instrumentation; and applications of supercritical fluid extractions

Presenters:
Les Myers, ISCO, Inc.; Steve Hut, Hewlett-Packard; Dale Felix, Dionex Corporation; Joseph M. Levy, Suprex Corporation

WORKSHOP

Workshop on Juice Analysis

Chairman: Carla Barry, Agriculture Canada

Full day workshop featuring current topics and new techniques given by experts in the field. Format will employ small groups and instructors using posters rather than instrumentation to illustrate examples.

Matrix Approach. Allan Brause, Analytical Chemistry Services of Columbia
Statistical Analysis (Pattern Recognition/Neural Network). Richard H. Albert, U.S. Food and Drug Administration
Enzymatic Techniques. Hans Hoffsomner
SNIF NMR Analysis. Hugh McManus, EUROFINS Laboratories, Inc.
Stable Isotope Analysis. Dana Krueger, Krueger Food Laboratories, Inc.
Carbohydrate Analysis. Nicholas H. Low, University of Saskatchewan
Chiral Chromatography. Thomas A. Eisele, Tree Top, Inc.
Trace Metals. Karen Barnes, Perkin Elmer
Capillary Electrophoresis. Paul Cancalon, Florida Department of Citrus
Anthocyanins. Bob Durst, Oregon State University
High Performance Liquid Chromatography, Polyphenolics. Sanford Kirksey, Procter & Gamble

FORUM

Methods for Antibiotics and Drugs in Feed

Chairman: Mark Coleman, Lilly Research Laboratories

Presentations:
Comparison of Zone Variation within CTC, OTC, Neomycin, and Bacitracin Assays. Mary Lee Hasselberger, Nebraska Department of Agriculture

Continuing Studies on a Procedure to Extract Deconquinate From Feeds. Kendrick Albert and Robert L. Smallidge, Office of the Indiana State Chemist

Monesin HPLC Collaborative Study -- Preliminary Report. Mark Coleman, Eli Lilly and Company

Update on FDA Feed Method Trial. Mary Leadbetter, U.S. Food and Drug Administration

Group Discussion Topics:
Compounds that Interfere with an Assay
How are Control Feeds Reported?

Progress Reports:
Apramycin. Hussein Ragheb, Office of the Indiana State Chemist
Bambermycins. Mustapha A. Mustapha, Barrow Agee Laboratories
Chloretracycline. Mary Lee Hasselberger, Nebraska Department of Agriculture
Tylosin. Mark Coleman, Eli Lilly and Company
Lasalocid. Paul Duke, Hoffmann-La Roche
Sulfadimethozine and Ormetoprim. Paul Duke, Hoffman-La Roche
Narasin. Mark Coleman, Eli Lilly and Company
Oxytetracycline. Mary Lee Hasselberger, Nebraska Department of Agriculture
Sulfamethazine. Robert Smallidge, Office of the Indiana State Chemist
Question and Answer Session -- Open Discussion

Monday • September 12, 1994

Opening Session

The Opening Session marks the official beginning of the 108th AOAC INTERNATIONAL Annual Meeting. The following is a tentative schedule of events:

Opening Remarks: Arvid W. Munson, President

President's Address: Arvid W. Munson

Presentation of Awards:
Employee Service Awards
Associate Referee of the Year
General Referee of the Year
Collaborative Study of the Year
Fellows of the AOAC

Presentation of the Harvey W. Wiley Award to W. Harvey Newsome: Arvid W. Munson. The Address of the 1994 Harvey W. Wiley Award Winner, The Evolution of Pesticide Residue Methodology, will be given in the Harvey W. Wiley Award Symposium on Pesticides in the Diets of Infants and Children, on Monday afternoon.

Featured Speaker: Ellen Haas, U.S. Department of Agriculture

Opening of the Exhibition

SYMPOSIUM

Harvey W. Wiley Award Symposium: Pesticides in the Diets of Infants and Children

Chairman: Stanley Katz, Rutgers University

Keynote Address: **The Evolution of Pesticide Residue Methodology,** W. Harvey Newsome, 1994 Harvey W. Wiley Award recipient, Health Canada

Additional resentations to be announced.

SYMPOSIUM

Automated Methods I

Chairmen: Allen S. Carman, U.S. Food and Drug Administration; John E. Gilbert, U.K. Ministry of Agriculture, Fisheries and Food

Overview of Multidimensional Methods for Automated Analysis. Martin Shepherd, U.K. Ministry of Agriculture, Fisheries and Food

Automated Analysis of Mycotoxins Using an ASPEC System. John E. Gilbert, U.K. Ministry of Agriculture, Fisheries and Food

Additional presentations to be announced.

TECHNICAL POSTER SESSION

Commodity Foods Products

Topics include: Cereals and Cereal Products; Seafoods; Dairy Chemistry; Fruits and Fruit Products; Meat, Poultry and Products; Processed Vegetable Products; Chocolate and Cacao Products; General Topics

Cereals and Cereal Products

Classification of Grains by Quality Using Chemical Sensors. Mel W. Finday, J.R. Stetter and W.M. Penrose, Transducer Research Inc.

Determination of Bromate in Baked Goods Using Reversed-Phase HPLC with a Postcolumn Reaction. Charles R. Warner, Daniel H. Daniels, Frank L. Joe, Jr., Benjamin J. Canas and Gregory W. Diachenko, U.S. Food and Drug Administration

Quantitative and Qualitative Comparisons of Acidic Materials from Cereal Extracts of the AOAC and ISO Fat Acidity Methods. O. Adolfsson and Ralph H. Lane, University of Alabama

Sample Preparation Studies on Wheat and Barley Prior to Deoxynivalenol Analysis. Tom Romer, Susan Hillermann and Jim Glamann, Romer Labs, Inc.

Analysis of Bioactive Substances in Flax. Paul Kolodziejczyk and Myles Marianchuk, POS Pilot Plant Corporation

Determination of Phosphine Residues in Cereal Grains and Selected Commodities by Ion Chromatography. Marvin Carlson and Richard D. Thompson, U.S. Food and Drug Administration

General Referee Report: Ralph H. Lane, University of Alabama

Seafoods

Collaborative Study: Determination of Histamine by Capillary Electrophoresis. Barry Mopper, U.S. Food and Drug Administration

Pattern Matching and Pattern Recognition Techniques Applied to Fish Speciation for Eventual Implementation in the Regulatory Setting. Brad Tenge, Walter E. Hill, Fredrick S. Fry, Ngoc-Lan Dang, Paul Trost, James D. Barnett, Patricia Rogers, Warren Savary and Marleen M. Wekell, U.S. Food and Drug Administration

Tripolyphosphates Orthophosphate and Pyrophosphates in Fish. Claude Desjardins, Fisheries and Oceans Canada

Long-Term Monitoring and Residue Component Pattern of Chlordanes in Short-Necked Clam from Tokyo Bay, Japan. Tomoyuki Miyazaki, Tsuneo Hashimoto, Tsutomu Nakazima, Yataro Kokubo and Tatsunori Yamagishi, The Tokyo Metropolitan Research Laboratory of Public Health

Categorization of Seafood Quality Using Several Decomposition Indices. Ellis Christopher, Mary Lou Silva, Rhode Island Department of Health; Chong M. Lee, University of Rhode Island

Using the Ammonia Ion Selective Electrode to Screen Raw Fish Quality. Ellis Christopher, Mary Lou Silva, Rhode Island Department of Health; Lori F. Pivarnik, University of Rhode Island

Analysis of Polycyclic Aromatic Hydrocarbon Concentrations in Lobster Digestive Gland for Time Trends and Compliance with Food Standards. John Uthe and R.K. Misra, Fisheries and Oceans Canada

GC/MS Confirmation of Leucomalachite Green, a Metabolite of Malachite Green, on Catfish (*Ictalurus Punctatus*) Tissues. Sherri B. Turnipseed, Jose E. Roybal and Austin R. Long, U.S. Food and Drug Administration; Jeffrey A. Hurlbut Metropolitan State College of Denver

Screening and Survey of Oxytetracycline in Farm Raised Catfish Using the Charm Test and HPLC Receptorgram. Joan Lawton Scheemaker, E. Zomer and S.E. Charm, Charm Sciences, Inc.

Liquid Chromatographic Determination of Sulfadiazine in Salmon Using Post-Column Derivatization and Fluorescence Detection. Theresa A. Gehring, Larry G. Rushing and Harold C. Thompson, Jr., U.S. Food and Drug Administration

Dairy Chemistry

Determination of Lactose in Dairy Products Using High Performance Anion Exchange Chromatography. John Mullin, Agriculture Canada

Determination of the Fat Content of Cream by the Modified Monjonnier Ether Extraction Method: Collaborative Study. David M. Barbano, Joanna M. Lynch and James R. Fleming, Cornell University

Difference in Cream Fat Test Results between the Modified Monjonnier Ether Extraction and Babcock Methods. Joanna M. Lynch, David M. Barbano and James R. Fleming, Cornell University

General Referee Report: Robert L. Bradley, University of Wisconsin-Madison

Fruits and Fruit Products

A Procedure for Detecting Grape Skin Extract as an Adulterant in Red Grape Juice Concentrate. Roger D. Williams, James L. Kutschinski, Steven A. Kupina and Anil J. Shrikhande, Heublein Inc.

Determination of D-Malic Acid in Apple Juice by HPLC: Collaborative Study Results. Thomas A. Eisele, Tree Top Inc.

New Approaches to Detecting Fruit Juice Adulteration. Jill Jekot, Andrew Woodruff, John Statler and Alan Henshall. Dionex Corporation

A Rapid SPE Prep for the Detection of BMIS in Fruit Juices. Linda L. Kline, Catherine L. Knapp and Nicholas W. Hether, Gerber Products Company

Apple Juice Authenticity Analysis by HPLC-PAD and Capillary Gas Chromatography. Nicholas H. Low, University of Saskatchewan

Authenticity of Cranberry Products Including Nondomestic Varieties. Elia Coppola, Noreen English, Joanne Provost, Anne Smith and Joseph Speroni, Ocean Spray Cranberries, Inc.

General Referee Report: Carla Barry, Agriculture Canada

Meat, Poultry and Products

Examples of Off-Odors in Aged or Contaminated Foods Identified with GC-MS. Daniel C. Harring, Brooks Army Medical Center Veterinary Laboratory

Determination of Fat in Food Products by an Automated Soxhlet-Type Extraction. Evelyn E. Conrad and Kevin P. Kelly, ABC Instruments

Simultaneous Determination of Ascorbic, Dehydroascorbic, Isoascorbic, and Dehydroisoascorbic Acids in Meat-Based Food Products by LC with Post-Column OPDA Fluorescence Detection. Sher Ali and Evan T. Phillippo, U.S. Department of Agriculture

A Combined Direct Saponification Method for the Determination of Cholesterol and Fatty Acids in Meat and Meat Products. Bruce Malone, Ken Ciriacy and Deborah Fields, Perdue Farms, Inc; Raymond Thompson, U.S Department of Agriculture

Automated Soxhlet Extraction, Cleanup, and Concentration for Chlorinated Pesticides in Low-Fat Meats. Nancy L. Schwartz, Evelyn E. Conrad and Kevin P. Kelly, ABC Instruments

Meat Product Proximate Results and Standard Deviations: Summary of a Check Sample Program. Pamela Coleman, Webb Technical Group, Inc.

Removal of Cyanide from the Titrimetric Method for Calcium in Mechanically Deboned Meat. Pamela Coleman, Webb Technical Group, Inc.

Influence of Sample Preparation and Sampling on NIT Analysis of Fat and Moisture on Ground Beef. William R. Windham, Kurt C. Lawrence and Franklin E. Barton, II, U.S. Department of Agriculture

Replacement of Mercury Catalyst in the Tecator Kjeltec Nitrogen/Protein Determination. George W. Greene, Lewis Hogarth, Gregory A. Shabdue, Carla A. Smith and Melissa D. Tuel, Abbott Laboratories

General Referee Report: David Soderberg, U.S. Department of Agriculture

Processed Vegetable Products

Determination of Nitrate in Vegetables by Continuous Flow: Collaborative Study. Paul R. Beljaars, Remmelt Van Dijk and Geertruida Van Der, Inspectorate for Health Protection

General Referee Report: Thomas R. Mulvaney, U.S. Food and Drug Administration

Chocolate and Cacao Products

General Referee Report: John Flanyak, EJ Brach, Inc.

General Topics

Quality Control Methods with Special Factor to Determine Food Adulteration. M. M. Zahid Shah Taimuru and Kishwar Shabina, Karachi Metropolitan Corporation

An Automated pH Workstation for the Food Industry. James M. Zdunek, Kraft General Foods

TECHNICAL POSTER SESSION

Food Nutrition

Topics include: Dietary Fibers; Infant Formula and Medical Diets; Fats and Oils; Minerals; Sugar and Sugar Products; Vitamins and Other Nutrients; General Topics

Dietary Fibers

Importance of Sample Preparation and Enzyme Treatments in the Determination of Dietary Fibers in Dried Legumes. Roger Mongeau and R. Brassard, Health Canada

Determination of Total Dietary Fiber in Foods and Food Products Using a Single Enzyme, Enzymatic-Gravimetric Method: Collaborative Study. Betty W. Li, U.S. Department of Agriculture

Determination of Soluble and Insoluble Dietary Fiber in Psyllium Products. Sungsoo Lee, F. Rodgriguez, M. Story and E. Farmakalidis, Kellogg Company; Leon Prosky, U.S. Food and Drug Adminstration

General Referee Report: Leon Prosky, U.S. Food and Drug Administration

Infant Formula and Medical Diets

Development of Stable Isotope Dilution Mass Spectrometry Methodology to Characterize SRM-1544 Fatty Acids in Diet Composite. Wayne R. Wolf and Michael Welch, U.S. Department of Agriculture

Determination of Niacin in SRM-1846 Infant Formula by Anion-Exchange HPLC. Dennis E. LaCroix and Wayne R. Wolf, U.S. Department of Agriculture

General Referee Report: Martin Bueno, U.S. Food and Drug Administration

Fats and Oils

Quality Evaluation and Compositional Studies of the Major and Minor Lipid Constituents of some Edible African Seed Oils. S.O. Yeboah, University of Botswana

Determination of Fat and Fatty Acids for Compliance with NLEA—Comparison with Traditional Methodology. Sandy Bailey, Gloria Gates, Deborah Marcuson and Philip J. Oles, Lancaster Laboratories

Liquid Chromatography Analysis of Sucrose Polyester in Vegetable Oil Blends Using Evaporative Light Scattering Mass Detection. William Chase Jr., Casimir C. Akoh and Ronald R. Eitenmiller, University of Georgia

A New Approach to Gas Chromatographic Method Development of Fatty Acid Methyl Ester Analysis. Michael J. Feeney, Resteck Corporation

Aldehyde Content for the Determination of Rancidity with Comparison to TBA Assay. Jack Cappozzo, Michael Pendleton and Grove Downers, Armour Swift-Eckrich, Inc.

General Referee Report: David Firestone, U.S. Food and Drug Administration

Minerals

To be announced

Sugar and Sugar Products

Carbohydrate Analysis Using Resin Based HPLC Columns. Elwood Doughty, Tracy Ascah and Roy Eksteen, Supelco Inc.

Malic Acid as Internal Standard for Authentification of Maple Syrup by Isotopic Ratio Mass Spectrometry. Real Paquin, Michel Bilodeau, Vincent Marquis, Luidgi Morais and Catherine Laplante, Agriculture Quebec; Marcel Baril and Daniel Michaud, Universite Laval

Comparison of Sugar Methodologies. Mary K. Krogull, James T. Wehrmann and Timothy Young, Hazleton Wisconsin

Analysis of Fructooligosaccharides from Jerusalem Artichoke and Chicory. John Mullin, Agriculture and Agri-Food Canada

Identification of Residual Tetracyclines in Honey by Frit FAB LC/MS Using a Volatile Mobile Phase. Oka Hisao, Ikai Yoshitomo and Hayakawa Junko, Aichi Prefectural Institute of Public Health; Asukabe Hirohiko and Suzuki Makoto, Meijo University; Hime Rurko, Kumamoto Prefectural Institute of Public Health; Horie Masakazu, Saitama Institute of Public Health; Nakazawa Hiroyuki, The National Institute of Public Health; and James D. MacNeil, Agriculture Canada

Determination of Sugar and Inorganic Ions in High Fat Foods by Ion Chromatography Combined with Supercritical Fluid Extraction. Jill Jeknot, Bruce Richter and Alan Henshall, Dionex Corporation

General Referee Report: Margaret A. Clark, Sugar Processing Research, Inc.

Vitamins and Other Nutrients

Automated Method for Extraction of 1-25 D Vitamin D. Douce Philippe and M. Puyfaucon, C.E.R.M.A.

Quantitative HPLC Determination of Vitamin C in Meats, Fruits, and Vegetables. Jack Cappozzo, Teck Tiong and Christi Hruskovich, Armour Swift-Eckrich Inc.

General Referee Report: Mike J. Deutsch, U.S. Food and Drug Administration

General Topics

Survey of Nutrient Measurements by Commercial Labs. Carol Davis, Joanne Holden, Wayne R. Wolf and Gary Beecher, U.S. Department of Agriculture

Meeting the Analytical Challenges of the Nutrition Labeling Education Act of 1990. Karen W. Barnes, Ebenezer Deborah and Uwe Volikopf, Perkin Elmer Corporation

New Classification of Methods of Analysis of Food Based on Horrat and Skurrat. Igor M. Skurikhin, Institute of Nutrition of Russian Academy of Medical Sciences (RAMS)

Assay of Menus as Part of Multi Center Clinical Feeding Trial: Comparison of Calculated and Assayed Total Fat Content and the Role of Quality Control. Katherine M. Phillips, Kent K. Stewart and Catherine Champagne, Virginia Polytechnic Institute

Estimated Fat and Volatile Liquid Contents of Ready to Eat Foods. J.L. Daft, U.S. Food and Drug Administration

Tuesday • September 13, 1994

SYMPOSIUM

Automated Methods II

Chairmen: Allen S. Carman, U.S. Food and Drug Administration; John E. Gilbert, U.K. Ministry of Agriculture, Fisheries and Food

Overview on Development of Standardized Automated Analysis Systems. Gary Kramer, National Institute of Standards and Technology

Robotic Systems for Analysis of Mycotoxins in Foods. Allen S. Carman, U.S. Food and Drug Administration

Additional presentations to be announced.

SYMPOSIUM

Analytical Methods for Herbal Products

Chairmen: Joseph M. Betz, U.S. Food and Drug Administration; Ylva Daveby, The Swedish University of Agricultural Science

Analytical Pharmacognosy: Macro/Micro/TLC Analysis of Plants. Ara Der Marderosian, Philadelphia College of Pharmacy and Science

Deducting the Identity of Commercial Plants Products—An Analytical Challenge. Dennis V.C. Awang, MediPlant

Product Development Strategies Based on Analytical Chemistry of Active Constituents of Garlic. Larry D. Lawson, Murdock Healthcare

Quality Assurance in the Herb Industry. Alvin Segelman, Nature's Sunshine

Determination of Known Toxic Components of Herbal Products: Pynolizidine Alkaloids. Russell J. Molyneux, U.S. Department of Agriculture

Analysis of Herbal Products in Response to Toxicological Episodes: Chaparral. William R. Obermeyer, U.S. Food and Drug Administration

SYMPOSIUM

Analytical Methods for the Determination of Economic Adulteration of Foods I

Chairman: Carla Barry, Agriculture Canada

Meats (Species Identification). Bruce Ritter, Elisa Tech, Inc.

Olive Oil. J.B. Russell, Leatherhead Food Research Association

Strategies for Detection of Economic Adulteration in Dairy Products. Don Carpenter, Kraft General Foods

Detecting Economic Adulteration of Honey and Maple Syrup. Landis W. Doner, U.S. Department of Agriculture

Common Adulterants in Coffee and Methods for their Detection. D. Richard White, Procter & Gamble

SYMPOSIUM

Analytical Methods for Fermentation Products

Chairman: Stephen Gendel, U.S. Food and Drug Administration

Application of Conduction Microcalorimetry for Monitoring Microorganisms. Michael C. Flickinger, University of Minnesota

Biochemical and Biological Tests for the Control of Fermentations and Fermentation Drug Products, An FDA Perspective. Yuan H. Chiu, U.S. Food and Drug Administration

New Analytical Methods for the Characterization of Proteins Produced by Fermentation. William Hancock, Analytical Medical Laboratory, Hewlett-Packard Company

Use of Capillary Gas Chromatography and Mass Spectroscopy to Determine Ethanal Stillage and Vegetable Oil Soapstock Compositions. Peter Reilly, Iowa State University

TECHNICAL POSTER SESSION

Microbiology and Extraneous Materials

Topics include: Food Microbiology-Dairy; Food Microbiology-Nondairy; Filth and Extraneous Materials in Foods and Drugs; Disinfectants; Drug Device Related Microbiology; Cosmetic Microbiology; Environmental Sanitation Microbiology; Analytical Mycology and Microscopy; Water Microbiology; General Topics

Food Microbiology-Dairy

Evaluation of a 22-Hour Detection Method for *Salmonella* in a Wide Range of Raw and Processed Foods. Dianne Kerr and Megan Ash, TECRA Diagnostics; Denise Hughes, Micotech Laboratories; Max Smith, CSIRO Meat Research; Cathy Fitzgerald, MIRINZ; Dawn Grassick, Australian Quality Egg Farms

Comparison of Sorbitol MacConkey Agar and Hemorrhagic Coli Agar the Recovery of *Escherichia Coli 0157.H7* from Selected High-Moisture Dairy Foods. Thomas B. Hammack, Peter Feng and Wallace H. Andrews, U.S. Food and Drug Administration

General Referee Report: J. Russell Bishop, University of Wisconsin-CDR, Madison

Food Microbiology-Nondairy

The Novel Combination of Uritcult® CRED/EMB Culture-Paddles® and Immunoblot Technology for the Isolation, Identification, and Enumeration of Protein A Positive *Staphylococcus Aureus*. J.H. Harris, S.E. Katz and S.J. Steiner, Cook College, Rutgers University

Comparison of the Crystal Enteric/Nonfermentor (E/NF) Identification System and Conventional Biochemical Procedures for the Identification of Enterobacteriaceae. Michael T. Knight, M.C. Newman, M.J. Benzinger, Jr., J.R. Agin, K.L. Neufang and J.L. Voet, Q Laboratories, Inc.

Comparison of the Petrifilm Dry Rehydratable Film and Conventional Culture Methods for the Enumeration of Yeast and Molds in Foods. Michael T. Knight, M.C. Newman, M.J. Benzinger, Jr. and J.R. Agin, Q Laboratories, Inc.

***Listeria* in Food Products, VIDAS *Listeria* Assay.** Michael T. Knight, M.C. Newman, M.J. Benzinger, Jr. and J.R. Agin, Q Laboratories, Inc.

Tecra *Listeria* Visual Immunoassay Method for the Detection of *Listeria* in Foods: Collaborative Study. Michael T. Knight, M.C. Newman, M.J. Benzinger, Jr. and J.R. Agin, Q Laboratories, Inc.; Megan Ash and Paul Simms, Bioenterprises Pty. Ltd.

Polymerase Chain Reaction for the Detection of *Listeria Monocytogenes*. Janet B. Gray and Chryste D. Best, U.S. Food and Drug Administration

Evaluation of API *Listeria* Identification Test Strips. Judy Heisick, U.S. Food and Drug Administration

Enzymatic Enhancements of *Salmonella* Recovery from Guar Gum. Rene Miguel Amaguana, Patricia S. Sherrod, Thomas S. Hammack, Geraldine A. June and Wallace H. Andrews, U.S. Food and Drug Administration

Comparative Efficacy of Modified Atmosphere Enrichment and Oxyrase Enrichment Procedures for Isolation of Campylobacter Species from Inoculated Foods. Tony T. Tran, U.S. Food and Drug Administration

Growth and Toxin Production by *Clostridium Botulinum* in Sliced Raw Potatoes in MAP with and without $NaHSO_3$. Haim M. Solomon, E. Jeffery Rhodehamel and Donald A. Kautter, U.S. Food and Drug Administration

Identification of Staphylococcal Enterotoxin by Monovalent Capture/Polyvalent Antibody Enzyme Conjugate ELISA: An Interlaboratory Evaluation. Reginald W. Bennett, U.S. Food and Drug Administration

Comparative Study for Detection of *Salmonella* in Foods by Conventional Culture Procedure and a Modified Deoxyribonucleic Acid Hybridization Method. Gregory W. Durbin, Karen M. Keough, Gwen N. Reynolds, GENE-TRAK Systems

General Referee Report: Wallace H. Andrews, U.S. Food and Drug Administration

Filth and Extraneous Materials in Foods and Drugs

Comparison of Reagent and Recrystallized Reagent in the Xanthydrol/Urea Method. Patricia A. Valdes and George C. Ziobro, U.S. Food and Drug Administration

General Referee Report: Jack L. Boese, U.S. Food and Drug Administration

Disinfectants

Development of Microtiter Plate Spore Carrier for the Automation of Disinfectant Testing. Judy Heisick, U.S. Food and Drug Administration

General Referee Report: Aram Beloian, U.S. Environmental Protection Agency

Drug Device Related Microbiology

General Referee Report: Ana M. Placencia, U.S. Food and Drug Administration

Cosmetic Microbiology

General Referee Report: Anthony D. Hitchins, U.S. Food and Drug Administration

Environmental Sanitation Microbiology

General Referee Report: Stephen Edberg, Yale University School of Medicine

Analytical Mycology & Microscopy

General Referee Report: Stanley Cichowicz, U.S. Food and Drug Administration

Water Microbiology

General Referee Report: Alfred P. DuFour, U.S. Environmental Protection Agency

General Topics

Estimation of Microbial Biomass in Solid State Fermentation System. Shen Rui, Huaiyin Industrial College; Ji Yueqin, Anhi Agricultural College

TECHNICAL POSTER SESSION

Natural Toxins

Topics include: Seafood Toxins; Ochratoxins; Plant Toxins Fumonisins; Mycotoxins

Seafood Toxins

Analysis of Marine Toxins by Capillary Electrophoresis. Michael A. Quilliam, National Research Council Canada

Ochratoxins

Determination of Ochratoxin A in Beer. Peter M. Scott and Shriniwas R. Kanhere, Health Canada

Plant Toxins

Determination of Lobeline in *Lobeliainflata* Containing Dietary Supplements by Capillary Electrophoresis. Joseph M. Betz, U.S. Food and Drug Administration

Saponin Content of Dehulled Peas (*Pisum* sativum L.). Yiva Daveby, Swedish University of Agricultural Science; Joseph M. Betz and W. Obermeyer, U.S. Food and Drug Administration

General Referee Report: Joseph M. Betz, U.S. Food and Drug Administration

Fumonisins

Determination of Hydrolyzed Fumonisin B_1 in Alkali-Processed Corn Foods. Peter M. Scott and Gulliaume A. Lawrence, Health Canada

Mycotoxins

Detection and Quantitation of T-2 Mycotoxin by Enzyme-Linked Immunosorbent Assays Utilizing Toxin Specific Polyclonal Antibodies Raised in Chicken. Danuta Kierek-Jaszczuk, Ronald R. Marquardt, Andrew A. Frohlich, James R. Clarke and Hao Xiao, University of Manitoba; David Abraham, Agriculture Canada

Quantification of Ochratoxin A in Swine Kidneys by Enzyme-Linked Immunosorbent Assay. James R. Clark, Ronald R. Marquardt, Andrew A. Frohlich and Randy J. Pitura, University of Manitoba

Factors Affecting the Production of Hydroxylated Ochratoxin A in Rats. Andrew A. Frohlich, Hao Xiao, Darren H. J. Kirsch and Ronald R. Marquardt, University of Manitoba

Isolation and the Structural Elucidation of Ochratoxin A Metabolites in Fermented Wheat. Hao Xiao, Andrew A. Frohlich and Ronald R. Marquardt, University of Manitoba

Structure-Activity Relationship and Interactions Among Trichothecenes as Assessed by Yeast Bioassay. Srinivasa Madhyastha, Ronald R. Marquardt and Andrew A. Frohlich, University of Manitoba; David Abramson, Agriculture Canada

Rapid Detection of Fumonisins in Corn by Membrane-Based Immunochemical Assay. Elisabeth Schneider, Ewald Usleber and Erwin Martlbauer, University of Munich

Occurrence of Fumonisins in Corn-Based Foods from the German Market. Christian Schlichtherle, Ewald Usleber and Erwin Martlbauer, University of Munich

Capillary Electrophoresis as a Tool for Mycotoxin Analysis: The Fumonisins. Chris M. Maragos, U.S. Department of Agriculture

Analyses of Fumonisin Contaminated Corn from International Sources Using Immunoaffinity Columns. Mohamed Abouzied, Scott Askegard, Chuck Bird and Brinton M. Miller, Neogen Corporation

Occurrence of Mycotoxins in Mexican Corn. Juan Carlos Medina, Joel Munoz, Eliezer Castillo and Miguel Romero, NUTEK S.A. de C.V.

Large Scale Isolation and Purification of Fumonisin B_1. Robert M. Eppley, Michael Stack, Fredrick Thomas, Samuel Page and Ronald Plattner, U.S. Food and Drug Administration

Veratox® AST: A Quantitative Aflatoxin ELISA with One Control Sample. Chuck Bird, Dan Shimer and Deborah Sorenson, Neogen Corporation

Determination of Fumonisin by In-Line, Post-Column Fluorometric Technic. Makoto Miyahara, Hiroshi Akiyama Masatake Toyoda and Yukio Saito, National Institute of Health Science

Affinity Column Cleanup and Direct Fluorescence Measurement of Aflatoxins in Spices. Thomsen J. Hansen and Nancy A. Zabe, VICAM

Survey of Deoxynivalenol in U.S. Wheat and Barley Crops by Enzyme-Linked Immunosorbent Assay. Mary W. Trucksess, U.S. Food and Drug Administration

General Referee Report: Mary Trucksess, U.S. Food and Drug Administration

TECHNICAL POSTER SESSION

Environmental Quality

Topics include: Inorganic Methods; Organic Methods; Cooperative Studies; General Topics

Inorganic Methods

To be announced

Organic Methods

Determination of Iodine and Bromide in Waters and Brines by ICPAES. Kim Anderson, University of Idaho

A Performance Evaluation of Solid Phase Extraction Disks from Various Manufacturers. Alice Kells, Purdue University

Multiresidue Pesticide Extraction of Soil Using Phase Emore Disk. Debbie Schwedler, Purdue University

Extraction of Carbendazim in Soil by Supercritical Extraction and Analysis by HPLC and ELISA. Rodney J. Bushway and Lance B. Paradis, Hall University; Athos Rosselli, Suprex; Barbara Young, Millipore Corporation

Automated Soxhlet Extraction, Cleanup, and Concentration for Chlorinated Pesticides in Low-Fat Meats. Nancy L. Schwartz, Evelyn E. Conrad and Kevin P. Kelly, ABC Instruments

Automated Extraction of Priority Pollutants from Aqueous Environmental Samples. Loren C. Schrier, Evelyn E. Conrad and Kevin P. Kelly, ABC Instruments

Determination of Fat in Food Products by an Automated Soxhlet-type Extraction. Evelyn E. Conrad and Kevin P. Kelly, ABC Instruments

Determination of Atrazine in Water by Magnetic Particle Immunoassay: Collaborative Study. Mary C. Hayes and David P. Herzog, OHMICRON Corporation

New Methods for Automated Analysis of Trace Contaminants in Drinking Water. Ruth Ann Kiser, Robert Joyce and Harprut Dhillon, Dionex Corporation

Applications of Microwave-Assisted Extraction of Organic Compounds from Soil/Sediment Samples. Viorica Lopez-Avila, Richard Young, Janet Benedicto and Robert Kim, Midwest Research Institute; Werner F. Beckert, U.S. Environmental Protection Agency

Simple and Rapid Analysis of Dissolved Oxygen in Pure Water by High-Performance Liquid Chromatography with Electrochemical Detection. Keiko Nakajima and Hiroyuki Nakazawa, The Kisarazu Public Health Center of Chiba Prefecture

General Referee Report: Viorica Lopez-Avila, Midwest Research Institute

Cooperative Studies

Analytical Method for the Determination of Botran and its Major Degradates in Soil. Stephen O. Jacobson, Kirk P. Payne, John Hatfield, Janelle Whitehouse and Peter A. Nelson, Compliance Services International

General Referee Report: Joseph R. Donnelly, Lockhead Engineering & Sciences Co.

General Topics

Computer Aids for Documenting GALP/ISO 9000. Leigh Richardson and Linda Vautrin-Hale, Telecation, Inc.

Can Field Contaminated Soil Be Adequately Simulated Using Fortification? Thomas F. Jenkins, Clarence L. Grant, Karen F. Myers and Erica F. McCormick, US Army CRREL

General Referee Report—Organics in Water. Viorica Lopez Avila, Midwest Research Institute

TECHNICAL POSTER SESSION

Drugs and Related Topics

Topics include: Drugs Residues in Animal Tissues; Cosmetics; Drugs I; Drugs II; Drugs III; Drugs IV; Drugs V; Diagnostics and Test Kits; Forensic Sciences; General Topics

Drug Residues in Animal Tissues

How can We Characterize and Optimize the Quality of Analytical Methods? W. G. de Ruig and H. Van der Voet, RIKILT-DLO

Determination of B-Agonoitz Residues Using LC-APCI/MS. Daniel R. Doerge, National Center for Toxicological Research

Determination of Ractopamine Residues in Biological Matrices — Recent Development Efforts. Michael P. Turberg, John W. Moran, John M. Rodewald, James M. Turner, William J. Barker and Mark R. Coleman, Lilly Research Laboratories

Determination of Tylosin by Microturbidimetric (96 Well Microplate) Assay. Alan L. Wicker, John W. Morgan, Daniel H. Mowrey and Mark R. Coleman, Lilly Research Laboratories

The Use of Isotope Enriched Internal Standards in Residue Analysis for Veterinary Drugs and Anabolic Compounds, Progress within the European Union. L.A. Van Ginkel, R.W. Stephany, H.J. Van Rossum and P.W. Zoontjes, National Institute of Public Health and Environmental Protection

Multi-Residue ELISA for Benzimidazole Anthelminthics in Liver. David L. Brandon, R.G. Binder, A.H. Bates and W.C. Montague Jr., U.S. Department of Agriculture

Determination of Monensin in Edible Bovine Tissues and Milk by HPLC. John W. Morgan, Mark R. Coleman, John M. Rodewald and James M. Turner, Lilly Research Laboratories

Production and Characterization of Group Specific Antibodies Against Unmodified Beta-Lactam Antibiotics. Susanne Kurz, Ewald Usleber and Erwin Martlbauer, University of Munich

Enzyme Immunoassay for the Detection of the Isoxazolyl, Penicillins Oxacillin, Cloxacillin and Dicloxacillin in Milk. Ewald Usleber and Erwin Martlbauer, University of Munich

Rapid, Simple Determination of Ceftiofur Residue in Bovine Milk by Ion-Paired Liquid Chromatography. Krystyna L. Tyczkowska, Arthur L. Aronson and Robert D. Voyksner, North Carolina State University

A Competitive Protein Binding Assay for Spectinomycin Using Particle Concentration Fluorescence Technique. Marjorie B. Medina, U.S. Department of Agriculture

A Method for the Determination of Thionin, a Metabolite of Methylene Blue, in Milk by VIS/LC. Jose E. Roybal, Robert K. Munns, David C. Holland and Austin R. Long, U.S. Food and Drug Administration; Jeffrey A. Hurlbut, Metropolitan State College of Denver

Determination of Flunixin in Milk Using Liquid Chromatography with GC/MS-SIM Confirmation. Heidi S. Rupp, David C. Holland, Robert K. Munns, Sherri B. Turnipseed and Austin R. Long, U.S. Food and Drug Administration

Determination of Lasalocid, Monensin, Narasin and Salinomycin in the Tissues of Food Animal by Liquid Chromatography. Geoff C. Gerhardt, Craig D.C. Salisbury and Harold M. Campbell, Agriculture and Agri-Food Canada

Liquid Chromatographic Determination of Streptomycin and Dihydrostreptomycin in Bovine Kidney Tissue Using Post-Column Fluorometric Detection. Frank Schenck, Badar Shaikh, U.S. Food and Drug Administration

Determination of Furosemide, a Diuretic, in Bovine Milk Using Liquid Chromatography with Fluorimetric Detection. Badar Shaikh, U.S. Food and Drug Administration

Enzyme Conjugates for Competitive Avermectin ELISA Test. Gene Davis, Chari Johnson, Mohamed Abouzied and Brinton M. Miller, Neogen Corporation

The Current Overview of Anthelmintics in Japan and their Residual Analysis in Foods. Kuzue Takeba and Yararo Kokubo, The Tokyo Metropolitan Research Laboratory of Public Health; Hisao Oka, Aichi Prefectural Institute of Public Health; Hiroyuki Nakazawa, The National Institute of Public Health; Joseph A. Settepani, U.S. Food and Drug Administration

Anabolics Residues in Bovines, Immunoassay Methods. Alberto Longhi and Alfredo M. Montes Nino, Xenobiotics SRL

Analysis of Tricaine and its Metabolites in Fish Tissues. Michael A. Quilliam, N. Ishida, F. Law and M.S. Yong. National Research Council Canada

Confirmation of Sulfadimethoxine in Porcine Muscle and Kidney. Shigeru Kawata, Kazuhiko Sato, Yuusuke Nishikawa, Rumiko Morita and Masuo Yaba, Yokohama City Meat Inspection Office; Hiroshi Kikawa, Toshio Hidaka and Keiko Ishii, Yokohama City Institute of Health; Masakazu Horie and Saitama Prefectural Institute of Public Health; Hiroyuki Nakazawa, The National Institute of Public Health

Matrix Solid Phase Dispersion Extraction and Liquid Chromatography Determination of Ivermectin Residues in Milk. Frank J. Schenck, U.S. Food and Drug Administration

Determination of Amphoteric B-Lactam Antibiotics in Animal Tissue Using Automated HPLC Cleanup. William A. Moats and Raida Harik-Khan, U.S. Department of Agriculture

Integration of B-Lactam Screening Kits with Automated HPLC Cleanup and Analysis. Raida Harik-Khan and William A. Moats, U.S. Department of Agriculture

General Referee Report: James D. MacNeil, Agriculture Canada

Cosmetics

Nonvolatile N-Nitrosamines in Cosmetics and their Raw Materials Using HPLC-Particle Beam Coupled with TEA and MS Detection. Stanley M. Billedeau, Jon G. Wilkes, Thomas M. Heinz and Harold C. Thompson, Jr., U.S. Food and Drug Administration

General Referee Report: Rhonda S. Bayoud, Mary Kay Cosmetics

Drugs I

To be announced

Drugs II

General Referee Report: Edward Smith

Drugs III

To be announced

Drugs IV

Progress in the Direct Resolution of Chiral Drugs Via HPLC. Charlotte A. Brunner, William M. Adams and Thomas D. Doyal, U.S. Food and Drug Administration

General Referee Report: Linda Ng, U.S. Food and Drug Administration

Drugs V

HPLC Determination of Alkali Salts of p-Amino Benzoic Acid and Salicylic Acid in Pharmaceutical Formulations: A Collaborative Study. Richard D. Thompson, U.S. Food and Drug Administration

General Referee Report: Thomas G. Alexander

Diagnostics and Test Kits

To be announced

Forensic Sciences

General Referee Report: Stanley M. Cichowicz, U.S. Food and Drug Administration

General Topics

Simultaneous Determination of Triamcinolone and Salicylic Acid in Pharmaceutical Preparation by HPLC. Erika Rosa Maria Kedor-Hackmann, Elisabeth Apparecide Dos Santos Gianotto and Maria Ines Rocha Miritello Santoro, Universidade de Sao Paulo

Application of Micellar Electrokinetic Capillary Chromatography to the Analysis of Some Nitrogenous Base Drugs. Robert Roos, U.S. Food and Drug Administration

First-Derivative Spectrophotometric Assay of Timololmaleate in Ophthalmic Solutions. Maria Ines Rocha Miritello Santoro, Hyun Sun Cho and Erika Rosa Maria Kedor-Hackmann, Universidade de Sao Paulo

Determination of Low Level Impurities in Pharmaceutical Products. Landy B. White and Terry A. Rooney, Thermo Separation Products

Wednesday ◆ September 14, 1994

SYMPOSIUM

Microbiology Series, Old Friends, New Enemies: Biomedical Waste Treatment Technology and Assessment Methodology I

Chairman and Presentations: To be announced.

SYMPOSIUM

Economic Adulteration II

Chairman: Carla Barry, Agriculture Canada

Vanilla Authenticity. Pat Hoffman, McCormick & Co.

Essential Oils & Spices. Tom Buco, Giveudan Roure Corporation

Flavours. Wilhelm Pickenhagen, EUROFINS Laboratories Inc.

Wines & Spirits. Dominique Tusseau, Champagne Research Institute

Determining Authenticity and Juice Content of Fruit Beverages. Ron Wrolstad, Oregon State University

SYMPOSIUM

Laboratory Safety I

Chairman: Dirk Shoemaker, Nebraska Department of Agriculture

Co-Chairman: Maire Walsh, State Laboratory

Pollution Prevention in the Laboratory. Howard Wilson, U.S. Environmental Protection Agency

New Methods Designed to Reduce Laboratory Waste. William L. Budde, U.S. Environmental Protection Agency

Environmental Regulation Affecting Laboratory Operations. Howard Wilson, U.S. Environmental Protection Agency

Solvent Recycling. Al Kakovich, Burlington Environmental

SYMPOSIUM

Safety II

Chairman: Dirk Shoemaker, Nebraska Department of Agriculture

AOAC Safety Considerations. Maire Walsh, State Laboratory

Safety Consideration in Lab Design. John Wiskerchen, U.S. Food and Drug Administration

OSHA Lab Standard. To be announced

Lab Ergonomics. Dave Sebesta, Washington Department of Labor and Industries

SYMPOSIUM

Biomedical Waste Treatment Technology and Assessment Methodology—Regulatory Roundtable II

This will continue the morning symposium.

Chairman and Presentations: To be announced.

TECHNICAL POSTER SESSION

Pesticide Residues and Related Topics

Topics include: Multiresidue Methods; Metals and Other Elements; Organonitrogen Pesticides; Radioactivity; Organohalogen Pesticides; Organophosphorus Pesticides

Multiresidue Methods

Market Survey of Pesticide Residues in Baby Food Using the Charm Test for Organophosphates and Carbamates and GC/MS. D. Puopolo, S. Saul and E. Zomer, Charm Sciences Inc.

Possible Interference of Post-Harvest Chemicals in Residue Analysis by ELISA. Rodney J. Bushway and Lewis B. Perkins University of Maine; Barbara S. Young, Millipore Corporation; Titan S. Fan, ImmunoSystems

Determination of Diazinon in Lanolin by GC-AED and Confirmation by ELISA. Rodney J. Bushway and Lewis B. Perkins, University of Maine; Barbara S. Young, Millipore Corporation; B.S. Ferguson, ImmunoSystems

Application of Solid-Phase Microextraction to the Headspace Gas Chromatographic Analysis of Semi-Volatiles in Aqueous Matrices. Denis Page and Gladys Lacroix, Health Canada

Quantitation of Chlorpyrifos by a Magnetic Particle Enzyme Immunoassay. Fernando M. Rubio, Timothy S. Lawruk, David P. Herzog and Adriane M. Gueco, OHMICRON Corporation

ELISA's for Thiabendazole Residues in Produce Matrices. David L. Brandon, G.R. Binder, H.A. Bates and C.W. Montague, Jr., U.S. Department of Agriculture

Screening Procedure for Organochlorine and Organophosphorus Pesticide Residues in Milk Using Matrix Solid Phase Dispersion (MSPD) Extraction and Gas Chromatographic Determination. Frank J. Schenck and Roberta Wagner, U.S. Food and Drug Administration

General Referee Report: Leon D. Sawyer, U.S. Food and Drug Administration

Metals and Other Elements

Automated Microwave Sample Preparation with an Emphasis on Mercury. Nancy Hohlack, E. King, N. Barclay and N. Ferguson, CEM Corporation

Microwave Digestion of Foods for Inductively Coupled Plasma Atomic Emissions Spectrometry. Scott P. Dolan, U.S. Food and Drug Administration

Report on Metals and Other Elements — 1994. Milan Ihnat, Agriculture and Agri-Food Canada

Principles of Reference Material Chemical Characterization. Milan Ihnat, Agriculture and Agri-Food Canada

General Referee Report: Milan Ihnat, Agriculture and Agri-Food Canada

Organonitrogen Pesticides

Determination of Bitertanol in Bananas by HPLC Using Florescence Detection. Carl N. Syvertson and Harold Thompson Jr., U.S. Food and Drug Administration

Determination of Herbicide Residues: Simazine, Atrazine and Propazine in Catfish by High Performance Liquid Chromatography. David C. Holland, Robert K. Munns, Jose E. Royal and Jeffrey A. Hurlbut, U.S. Food and Drug Administration; Austin R. Long, Metropolitan State College of Denver

Solid Phase Extraction: An Alternate Approach to the Determination of Chlorinated Pesticide and Polychlorinated Biphenyls in Blood Serum. Virlyn W. Burse, J.W. Brock, A.R. Najam and M.P. Korver, Centers for Disease Control

The use of HPLC with Electrochemical Detection for the Determination of Sulfonylurea Herbicides. Ronald G. Luchtefeld and Robert A. Levine, U.S. Food and Drug Administration

General Referee Report: W. Harvey Newsome, Health Canada

Radioactivity

Determination of Cs-134 and Cs-137 in Food by Gamma-Ray Spectrometry: Collaborative Study. Paul R. Beljaars, Remmelt Van Dijk, Jos Geertsen and Hans Nootenboom, Inspectorate for Health Protection

General Referee Report: Edmond J. Baratta, U.S. Food and Drug Administration

Organohalogen Pesticides

General Referee Report: Bernadette McMahon, U.S. Food and Drug Administration

Organophosphorus Pesticides

To be announced

TECHNICAL POSTER SESSION

Pesticide Formulations

Topics include: Herbicides; Insecticides, Synergists and Repellents Formulations; CIPAC Studies; Fungicides and Rodenticides Formulations; General Topics

Herbicides

Acetanilide Herbicide Universal Determinations Using Electronic Pressure Controlled Wide-Bore Gas Chromatography. Eddy F. De Keyser, T.L. Cross and D.F. Tomkins, Monsanto Co.

General Referee Report: David F. Tomkins, Monsanto Co.

Insecticides, Synergists and Repellents Formulations

The Quantitation of Cyfluthrin in Liquid and Solid Formulations Using Reversed Phase HPLC: Collaborative Study. Don Harbin, Miles Inc.

General Referee Report: Benjamin Belkind, Sandoz Agro, Inc.

CIPAC Studies

General Referee Report: Alan R. Hanks, Office of the Indiana State Chemist

Fungicides and Rodenticides Formulations

General Referee Report: Richard Collier, CERIS

General Topics

Computerized Management of Pesticide Labels and Spray Records. Eric M. DeKuiper, Gerber Products Company; Elizabeth A. Magill, Crop Data Management Systems, Inc.

TECHNICAL POSTER SESSION

Feeds, Fertilizers and Related Topics

Topics include: Drugs in Feeds; Antibiotics in Feeds; Fertilizers and Agricultural Liming Materials; Veterinary Analytical Toxicology; Feeds; Tobacco; Nutrients in Soil

Drugs in Feeds

Liquid Chromatographic Determination of Dimetridazole in Feeds. Harold Campbell and Alma Jean Newman, Agriculture Canada

General Referee Report: Robert L. Smallidge, Office of the Indiana State Chemist

Antibiotics in Feeds

Determination of Neomycin in Animal Feeds at Therapeutic and Residue Levels by Capillary Gas Chromatography. Susan B. Clark, Robert J. Schmid, W. Douglas Rowe and Carolyn A. Geisler, U.S. Food and Drug Administration; Jeffrey A. Hurlbut, Metropolitan State College of Denver

Liquid Chromatographic Determination of Laidlomycin Propionate in Cattle Feeds: Interlaboratory Study. William S. Ward, Syntex Agribusiness; Mary G. Leadbetter, U.S. Food and Drug Administration

Liquid Chromatographic Dual Electrode Detection System for Benzilsochromequine Antibiotic in Feeds. Yacoob Haroon and J. Haroon, Hoffman La Roche

General Referee Report: Hussein S. Ragheb, Purdue University

Fertilizers and Agricultural Liming Materials

False Assumptions in the Official AOAC Method for Atomic Absorption Determinations of Feeds and Fertilizers. Peter F. Kane, Natalie F. Newlon and Ronald K. Sensmeier, Office of the Indiana State Chemist

Some Problems with Urea Determinations in Certain Fertilizers and a Proposed Solution. Thomas M. Parham, Jr., Arcadian Fertilizer, L.P.

Determination of Sulfur in Fertilizers by ICP-AES. Kim Anderson, University of Idaho

Skalar Analyzer for Fertilizer Analysis. Joseph E. Gliksman, IMC Agrico Company

General Referee Report: Peter F. Kane, Office of the Indiana State Chemist

Veterinary Analytical Toxicology

The Determination of Nitrogen in Urine Using Phyrochemiluminescence: A Collaborative Study. Kristi A. Boehm, Antek Instruments, Inc.

Determination of Homocysteine in Serum: Application of a Validation Method. Douce Philippe and M. Puyfacon, C.E.R.M.A.

General Referee Report: P. Frank Ross, U.S. Department of Agriculture

Feeds

Colorimetric Determination of Selenium as Selenite or Selenate in Mineral Premixes. Jeffrey A. Hurlbut, Metropolitan State College of Denver; Roger G. Burkepile, Philip J. Kijak and Carolyn A. Geisler, U.S. Food and Drug Administration

General Referee Report: William R. Windham, U.S. Department of Agriculture

Tobacco

General Referee Report: W. Wesley Weeks, North Carolina State University

Nutrients In Soil

General Referee Report: Charles Focht, Nebraska Department of Agriculture

TECHNICAL POSTER SESSION

Additives, Beverages and Food Process Related Analytes

Topics include: Food Additives; Flavors; Color Additives; Spices and Other Condiments; Nonalcoholic Beverages; Alcoholic Beverages; Economic Adulteration

Food Additives

Cyanogenic Glycosides in Food. William R. Obermeyer and Ryan Casey, U.S. Food and Drug Administration

Food Additives Yearly Progress Report. Thomas Fazio, U.S. Food and Drug Administration

Characterization of Volatiles in Food with Solid Phase Microextraction and Gas Chromatography. Zelda Penton, John Sullivan and Sue Ann Scheppers. Varian Chromatography Systems

Supercritical Fluid Chromatographic Determination of Chemical Residuals in Food Packaging. John E. Biles, Timothy H. Begley and Henry C. Hollifield, U.S. Food and Drug Administration

Supercritical Fluid Extraction Method for Nirtrosamines in Hams Processed in Elastic Rubber Nettings. Walter W. Fiddler, J.W. Pensabene, R.J. Maxwell, A.R. Lightfield and J.W. Hampson, U.S. Department of Agriculture

Determination of Low Nanogram/Milliliter Levels of Volatile Organic Compounds in On-Purpose and By-Product Hydrochloric Acids Used in Food Processing. Patricia J. Nyman, U.S. Food and Drug Administration

Comparative Analysis of Food Samples for Glutamic Acid via HPLC Using Phenylisothiocyanate Derivatization and Direct Injection. Daniel H. Daniels, Frank Joe, Jr. and Gregory W. Diachenko, U.S. Food and Drug Administration

Choice of Optimal Methods for the Detection of Added Whey Solids in Complex Products. Claudia Hischenhuber, Nestec S.A.

Determination of Polyamines in Foods by Liquid Chromatography with Automatic Sample Cleanup and On-Column Fluorescence Derivatization Using Column-Switching. Koichi Saito, Masakazu Horie and Yoshikazu Tokumaru, Saitama Institute of Public Health; Hiroyuki Nakazawa, National Institute of Public Health

New Methods for Determination of Organic Acids in Foods and Beverages. Andy Woodruff and Chris Pohl, Dionex Corporation

3M Disposable IR Cards for Qualitative Mid-Infrared Analysis. James Gagnon, 3M Company

Flavors

Improved HPLC Method for the Determination of Heterocyclic Aromatic Amines in Process Flavors. Gracia A. Perfetti, U.S. Food and Drug Administration

Supercritical Fluid Extraction of Flavor Components from Cheese and Other Food Matrices. David Knowles and Nathan Porter, Dionex Corporation; D. Ward, New Zealand Dairy Research Institute

Color Additives

Total Mercury Determination in Certifiable Color Additives by an Automated Technique. Nancy M. Hepp and Anne Cargill, U.S. Food and Drug Administration

Analysis of Food Dyes by Capillary Electrophoresis. Hiroyuki Nakozawa, Sumiko Sukuki, Mika Shirao, Momoyo Aizawa and Hirokuni Miyamato, The National Institute of Public Health; Yoshitomo Ikai and Hisao Oka, Aichi Prefectural Institute of Public Health; Hiroko Kishi, Kanagawa Prefectural Public Health Laboratories

Spices and Other Condiments

Liquid Chromatographic Method for the Determination of Capsaicinoids in Capsicums and their Extractives: Collaborative Study. Mark Parrish, McCormick & Company, Inc.

Analytical Methods for Ingredients in Garlic and Garlic Products. Emiko Mochizuki, Yamanashi Institute for Public Health

Nonalcoholic Beverages

Use of Ion-Pair Extraction for Liquid Chromatographic Detection of Acesulfame K, Aspartame Dulcin and Saccharin in Beverages. Pai-Wen Wu, National Laboratories of Food and Drugs

Alcoholic Beverages

Analyses of Vanillin, Ethyl Vanillin and Coumarin in Beverage Alcohol Products. Paul Schneider, Alan Reisig and Sumer Dugar, BATF Laboratory

Lead and Cadmium Leaching Into Beverage Alcohol Stored in Distinctive Liquor Containers. Sumer Dugar, Alan Reisig and Randy Dyer, BATF Laboratory

Economic Adulteration

Development of a Simple and Inexpensive Enzymatic Detection System for Lactose in Foods Employing a Microassay Format. Burton W. Blais, Agriculture and Agri-Food Canada

Thursday ◆ September 14, 1994

SYMPOSIUM

Analytical Methods for Processed Seafood Products

Chairman: Marleen M. Wekell, U.S. Food and Drug Administration

Introduction to Analytical Methods for Processed Seafood Products. Marleen M. Wekell, U.S. Food and Drug Administration

Sensory Methods for Analytical Processed Seafoods. James D. Barnett, U.S. Food and Drug Administration

Laboratory and Field Screening Methods for Seafood Decomposition Products. James Hungerford, U.S. Food and Drug Administration

Methods for Forensic Microbiological Evaluation of Processed Seafoods: PCR, Ribotyping. Karen Jinneman, U.S. Food and Drug Administration

Methods for Detection of Viruses in Processed Seafoods. Ronald L. Manger, Linda Leja and Marleen M. Wekell, U.S. Food and Drug Administration

Methods for Species Identification in Processed Seafoods. Bradley J. Tenge, Walter E. Hill, Ronald L. Manger, Ngoc-Lan Dang, Paula A. Trost, James D. Barnett and Marleen M. Wekell, U.S. Food and Drug Administration

SYMPOSIUM

Capillary Electrophoresis

Confronting the Analytical Challenges of the 90's. John E. Wiktrowicz, Applied Biosystems/Perkin Elmer Corporation

Application of Capillary Electrophoresis (CE) for the Determination of Pesticides in Agricultural Commodities. Maximillian M. Safarpour and Gerald L. Picard, American Cyanamid Company

Practical Application of Capillary Electrophoresis into Pesticide Residues. Alexander J. Krynitsky and Douglas M. Swineford, U.S. Environmental Protection Agency

Flexible Intravenous Microdialysis Probe and Capillary Electrophoresis for Blood Analysis in Freely Moving Animals. Norberto A. Guzman and R.W. Johnson, Pharmaceutical Research Institute

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- *Demonstrative Evidence
- *Cross Examination

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Cumulative Author Index

Abeyta, Jr, Carlos, 351
Abouzied, Mohamed M., 495
Ahmad, Shujaat, 11
Al-Haddad, Ameera, 437
Alanko, Timo A., 425
Albelda, Carmen, 74
Allen, John L., 553
Almond, Richard E., 466
Alvarez-Lueje, Alejandro, 768
Åman, Per, 703
Anderson, Kim A., 473
Arenas, Rene V., 710
Asp, Nils-Georg, 690
Bachmann, Claude, 774
Barbano, David M., 430
Barmark, Anna-Lena, 46
Beljaars, Paul R., 667, 672
Bennett, Reginald W., 357
Bennett, Glenn A., 501, 512
Biancardi, Claudio, 714
Blackwell, Barbara A., 506
Bogus, Edward R., 86
Boison, Joe O.K., 558, 565
Bolderdijk, R., 365
Boudra, Hamid, 517
Boulat, Olivier, 774
Bradley, Jr, Robert L., 488
Brands, Arie, 672
Branham, Bruce E., 525
Brinkman, Udo A. TH., 306
Bryce, John R., 403
Butler, Bernard P., 777
Cabanis, Jean C., 463
Calcagni, Albert, 577
Candrian, Urs, 617
Capar, Stephen G., 454
Cardozo, Maria S., 687
Cassini, Renee, 517
Cecchini, Giancarlo, 446
Chan, Wayne, 331
Chao, Yan-Zhong, 738
Chernomorsky, Simon, 756
Cieri, Ugo R., 758
Cirilli, Luciano, 446
Conacher, Henry B.S., 5
Corradini, Fulvio, 714
Cox, Maurice G., 777
Curiale, Michael S., 374, 602
Danielson, James W., 53
Davis, Paula K., 583
de Blas, O. Jimenez, 441, 722
de Jong, Gerhardus J., 306
De Smedt, J., 365
Decker, Eric A., 421
DeVries, Jonathan W., 690
Di Corcia, Antonio, 446
Dijk, Remmelt van, 667, 672
Doko, M. Bruno, 546
Dolan, Scott P., 454, 718
Dombrink–Kurtzman, Mary A., 512
Dowell, Floyd E., 107, 659
Dreas, John, 570
Dupuy, Jacques, 517
Dustman, Wendy A., 374
Dyer, Randolph H., 64
Eckner, Karl F., 374
Elkins, Edgar R., 411
Ellison, Stephen L.R., 777
Engelen, Adrianus J., 760
Epstein, Robert L., 570
Ericson, Jon F., 577
Falbo-Nelson, Maria T., 58
Fan, Chun-Lin, 738
Fanning, Luanne, 602
Fatima, Ismat, 11
Feldsine, Philip T., 58
Fernandez, Cecilia, 628
Firestone, David, 674, 677
Fleeker, James R., 86
Fleming, J. Richard, 430
Flowers, Russell S., 374
Font, G., 74
Forbes, Alastair B., 777
Fouler, Sophie G., 631
Friedman, Stephen B., 466
Furda, Ivan, 690
Gagnon, Michael R., 728
Garramone, Susan, 602
Gavalchin, Jerrie, 481
Gavlick, Walter K., 583
Gerhardt, Geoff C., 331, 334, 765
Ghijsen, Rudi T., 306
Giesbrecht, Francis G., 107, 659
Gofus, Jane E., 553
Gomez, B. De Leon, 722
Gonzalez, S. Vicente, 441, 722
Graham, Hadden, 703
Guinivan, Richard A., 728
Hägg, Margareta, 681
Hagler, Jr, Winston M., 107, 659
Hanks, Alan R., 8
Hannaby, Simon A., 777
Harris, Peter M., 777
Hawkins, George S., 454
Hayashi, Yuzuru, 338
Heeft, Fred C. van der, 760
Hennessy, Michael K., 102
Henry, Carolyn, 570
Herzog, David P., 86
Heuser, John R., 411
Hewitt, Alan D., 458, 735
Hitchins, Anthony D., 395
Ho, Koon-Bay, 591
Hodson, Susan M., 777
Holland, David C., 596
Holland, Kathleen P., 570
Holland, Patrick T., 79
Houwen-Claassen, Adrie A.M., 667
Hustead, David L., 58
Isaacs, Brandon, 473
Ishibashi, Mumio, 338
Itak, Jeanne A., 86
Jacobs, Richard M., 454
Johnson, Nelson A., 710
Juba, Jean H., 522
Katz, Stanley E., 481
Keng, Lily J. -Y., 558, 565
Kijak, Philip James, 34
Kirchhoefer, Ross D., 117, 587
Kitabatake, Naofumi, 631
Kumar, Luxmy, 591
Kuntom, Ainie, 67
Larroque, Michel, 463
Larsson, Kjell, 46
Lawrence, Guillaume A., 541
Le Bars, Joseph, 517
Le Bars, Pierrette, 517
Lee, Sungsoo C., 690
Lenk, Horst, 789
Lepper, Wendy, 602
Li, Betty W., 687
Long, Austin R., 344, 596
Low, Nicholas H., 695
Lund, Eric D., 416
Lüthy, Jürg, 617
Lynch, Martin J., 577
Lynch, Joanna M., 430
MacNeil, James D., 334, 565, 765
Malcolm, Colin P., 79
Mannan, Abdul, 11
Mañes, Jordí, 74
Mapes, James P., 466
Marchese, Stefano, 446
Marcheselli, Luigi, 714
Marchetti, Andrea, 714
Matsuda, Rieko, 338
McClure, Foster, 357
McDonald, Patricia P., 466
McIver, Dawn, 602

McNaughton, Donald E., 79
Meinertz, Jeffery R., 553
Mendez, J. Hernandez, 441, 722
Merrill, Jr, Alfred H., 533
Meyer, Rolf, 617
Milas, J., 365
Miller, J. David, 506
Moats, William A., 41
Möller, Gregory, 473
Mozola, Mark, 602
Mueller, Thomas C., 752
Mumma, Ralph O., 86
Munns, Robert K., 344, 596
Musser, Steven M., 628
Naidu, R. Raghava, 748
Neidert, Eli, 18
Nelson, Paul E., 522
Nesheim, Stanley, 637
Newell, Richard F., 637
Núñez-Vergara, Luis J., 768
Okrasinski, Jr, Joseph L., 102
Oxborrow, Gordon S., 53
Pang, Guo-Fang, 738
Parfitt, Charles H., 92
Park, Douglas L., 637
Parks, Owen W., 486
Peeler, James T., 53
Pestka, James J., 495
Picó, Yolanda, 74
Plakas, Steven M., 344, 596
Plattner, Ronald D., 525
Plunkett, Randall J., 454
Polak, Pavel, 488
Prentice, George A., 395
Preti, Carlo, 714
Prosky, Leon, 690
Qureshi, Iqbal Hussain, 11
Raju, R.V. Prabhakara, 748
Randsdorp, Peter H.G., 760
Rawling, Alan K., 782
Rice, Larry G., 522
Richard, John L., 501, 512
Richardson, Gary H., 623
Riley, Ronald T., 533
Ríos, Angel, 785
Robison, Barbara J., 374
Rodriguez, R. Seisdedos, 411, 722
Ross, P. Frank, 491, 522
Roybal, José E., 596
Rupp, Heidi S., 344
Salisbury, Craig D.C., 331, 334, 765
Samperi, Roberto, 446
Saschenbrecker, Peter W., 18
Savard, Marc E., 506
Schenck, Frank J., 102
Schmitt, Thomas M., 328
Schweizer, Thomas F., 690
Scott, Peter M., 541
Selisker, Michele Y., 86
Shantha, Nalur C., 421
Sherma, Joseph, 297
Sisson, Donald V., 623
Sjöberg, Anna-Maija K., 425
Smit, Ed L.C., 760
Smith, Erika B., 430
Sons, Terri, 602
Squella, Juan A., 768
Stack, Michael E., 628, 637, 655
Stehly, Guy R., 596
Stokes, Barry O., 623
Storey, Joseph M., 596
Sturm, Juan C., 768
Swallow, Kevin W., 695
Takeda, Yasushi, 338
Tan, Yew Ai, 67
Theander, Olof, 703
Thompson, Don W., 647
Tomero, Jose Antonio Sanchez, 722
Toothill, John R., 591
Tracy, Mark, 473
Trivedi, Alka B., 631
Trotman, Raymond B., 18
Trucksess, Mary W., 637, 655
Twedt, Robert M., 395
Valcárcel, Miguel, 785
Vian, Laurence, 463
Visconti, Angelo, 546
Vreuls, Jolan J., 306
Wagner, Roberta, 102
Waheed, Shahida, 11
Wang, Elaine, 533
Waples, Ivan R., 1
Westerlund. Eric, 703
Wetherington, June H., 351
Whitaker, Thomas B., 107, 659
Willian, William T., 752
Wu, Jeremy, 107, 659
Yuan, James T.C., 623
Zhao, Tie-Sheng, 738
Ziegler, Engelbert, 789

COMING IN THE NEXT ISSUE

CHEMICAL CONTAMINANTS MONITORING

Performance Evaluation of the Babcock and Ether Extraction Methods: 1989–1992—J. M. Lynch, D. Barbano, P. A. Healy, and J.R. Fleming

DRUGS, COSMETICS, AND FORENSIC SCIENCES

Improvement of Chemical Analysis in Antibiotics. XIX Analysis of Tetracycline Antibiotics in Milk by Liquid Chromatography and Thin-Layer Chromatography/Fast Atom Bombardment Mass Spectrometry—H.O. Oka, Y. Ikai, J.O. Hayakawa, K. Masuda, K.-I. Harada, and M. Suzuki

FOOD BIOLOGICAL CONTAMINANTS

Update on Validation of Microbiological Methods by AOAC INTERNATIONAL—W.H. Andrews

RESIDUES AND ELEMENTAL ANALYSIS

Gas Chromatographic Determination of Polychlorinated Biphenyls in Mussels from Galicia, Northwest Spain—E. Alvarez Piñeiro, J. Simal Lozano, and A. Lage Yusty

DRUGS, COSMETICS, FORENSIC SCIENCES

Determination of Malachite Green Residues in the Eggs, Fry, and Adult Muscle Tissue of Rainbow Trout (*Oncorhynchus mykiss*)

JOHN L. ALLEN, JANE E. GOFUS, and JEFFERY R. MEINERTZ
U. S. Fish and Wildlife Service, National Fisheries Research Center, PO Box 818, La Crosse, WI 54602-0818

Malachite green, an effective antifungal therapeutant used in fish culture, is a known teratogen. We developed a method to simultaneously detect both the chromatic and leuco forms of malachite green residues in the eggs, fry, and adult muscle tissue of rainbow trout (*Oncorhynchus mykiss*). Homogenates of these tissues were fortified with [^{14}C] malachite green chloride and extracted with 1% (v/v) acetic acid in acetonitrile or in methanol. The extracts were partitioned with chloroform, dried, redissolved in mobile phase, and analyzed by liquid chromatography (LC) with postcolumn oxidation of leuco malachite green to the chromatic form. LC fractions were collected every 30 s for quantitation by scintillation counting. Recoveries of total [^{14}C] malachite green chloride residue were 85 and 98% in eggs fortified with labeled malachite green at concentrations of 0.5 and 1.00 μg/g, respectively; 68% in fry similarly fortified at a concentration of 0.65 μg/g; and 66% in muscle homogenate similarly fortified at a level of 1.00 μg/g. The method was tested under operational conditions by exposing adult rainbow trout to 1.00 mg/L [^{14}C] malachite green chloride bath for 1 h. Muscle samples analyzed by sample oxidation and scintillation counting contained 1.3 and 0.5 μg/g total malachite green chloride residues immediately after exposure and after a 5-day withdrawal period, respectively.

Malachite green has been used as an effective treatment for external fungal and protozoan infections of fish since 1933 (1–4), but it was never registered for use on food fish (5). Malachite green belongs to the triphenylmethane class of dyes, some of which are animal carcinogens (6). Meyer and Jorgenson (7) demonstrated that malachite green caused significant development abnormalities when administered to eggs of rainbow trout (*Oncorhynchus mykiss*) and to pregnant New Zealand white rabbits (*Oryctolagus cuniculus*).

In animals, malachite green is reduced through biotransformation to its colorless form, leuco malachite green (8–10). The leuco form of malachite green is a precursor of the chemical during production and could be a contaminant in the commercially prepared dye. Although it has never been tested for carcinogenicity, leuco malachite green is structurally similar to classic aromatic amines that are carcinogens (11). To address concerns regarding the health risks associated with the use of malachite green required that a method be developed to monitor these residues in food fish tissues. Presently, the chromatic and leuco forms of other dyes can be determined simultaneously by liquid chromatography (LC) with electrochemical detection (11, 12). Bauer et al. (13) determined leuco and chromatic malachite green in a fish tissue sample by splitting the sample in half and oxidizing half of the sample with PbO_2. The entire sample was then analyzed for chromatic malachite green, and the amount of leuco malachite green in the sample was determined by the difference between the unaltered and oxidized subsamples. Chromatic malachite green and leuco malachite green have been analyzed by the LC system and detected by visible spectrophotometry after postcolumn oxidation of leuco malachite green to the chromatic form (see Figure 1) (14, 15). We, therefore, included postcolumn oxidation in our method to detect total malachite green residues in the eggs, fry, and adult muscle tissue of rainbow trout.

Experimental

Apparatus

(a) *LC system.*—Beckman (Beckman Instruments, Inc., Fullerton, CA) Model 110A pump, equipped with Beckman Model 210 injection valve and 50 μL fixed-loop injector.

(b) *LC column.*—Waters (Waters Chromatography, Div. of Millipore, Milford, MA) μBondapak C_{18} column, 300 mm × 3.9 mm id, particle size 10 μm.

(c) *Spectrophotometric detector.*—Waters (Waters Chromatography) Lambda-Max Model 481 spectrophotometer detector operated at 618 nm.

(d) *Chromatographic software.*—System Gold (Beckman Instruments, Inc., Arlington Heights, IL).

(e) *Postcolumn reactor.*—Stainless steel tube, 32 × 4 mm id, packed with 10% PbO_2 suspended in Celite 545 (PbO_2 is

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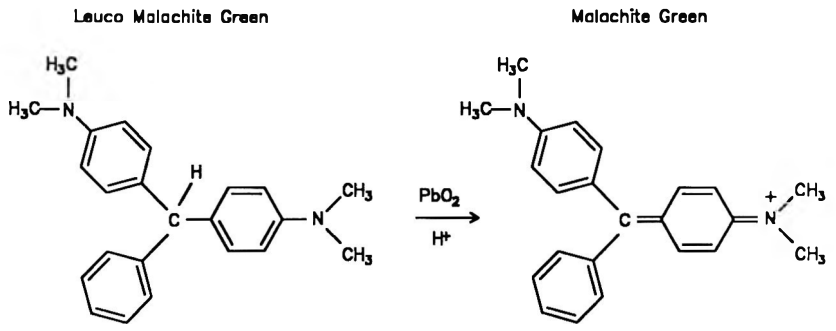

Figure 1. Oxidation of leuco malachite green to chromatic malachite green.

previously dry-mixed with Celite to give uniform mixture) and capped with 2 µm frits. As the reactor is being packed with PbO_2 in Celite, gently tap it to prevent the formation of voids. Place postcolumn reactor in line between the LC column and the spectrophotometric detector.

(**f**) *Fraction Collector.*—"Foxy" (Isco, Inc., Instrument Div., Lincoln, NE) fraction collector.

(**g**) *Homogenizer.*—Virtis (The Virtis Co., Gardner, NY) Hi-speed "45" homogenizer.

(**h**) *Blender.*—Waring (Waring Products Div., New Hartford, CT), or equivalent, equipped with a stainless steel cup.

(**i**) *Filtration columns.*—Baker (J.T. Baker Inc., Phillipsburg, NJ) Bakerbond spe* 6 mL disposable filtration columns.

(**j**) *Evaporator.*—Wheaton (Wheaton, A Div. of Wheaton Industries, Millville, NJ) Heidolph rotary evaporator.

(**k**) *Syringe filters.*—ACRO (Gelman Sciences, Inc., Laboratory/Diagnostics, Ann Arbor, MI) LC13 disposable filter assembly.

(**l**) *Sample oxidizer.*—Packard (Packard Instrument Co., Meriden, CT) Model D0306 biological sample oxidizer.

(**m**) *Liquid scintillation counter.*—Beckman (Beckman Instruments, Inc., Arlington Heights, IL) LS5801 liquid scintillation system.

Chemicals and Reagents

(**a**) *LC mobile phase.*—Mobile phase (flow rate 1.5 mL/min), consisting of 85 + 15 mixture of methanol to aqueous acetate buffer (0.05 M sodium acetate and 0.1 M glacial acetic acid in LC grade water).

(**b**) *Malachite green oxalate.*—Cat. No. 1264 (Eastman Kodak Co., Rochester, NY).

(**c**) *Leuco malachite green.*—Cat. No. 3620 (Eastman Kodak Co., Rochester, NY).

(**d**) *[Methano-^{14}C] malachite green chloride.*—Chemsyn Science Laboratories, Lenexa, KS.

(**e**) *Anhydrous acetic acid.*—Cat. No. 24, 124-5 (Aldrich Chemical Co., Inc., Milwaukee, WI).

(**f**) *Anhydrous sodium bicarbonate.*—Cat. No. S-8875 (Sigma Chemical Co., St. Louis, MO).

(**g**) *Sodium acetate (acetic acid, sodium salt, anhydrous).*—Cat. No. 24, 124-5 (Aldrich Chemical Co., Inc.).

(**h**) *Anhydrous sodium sulfate.*—Cat. No. 7757-82-6 (Fisher Scientific, Pittsburgh, PA).

(**i**) *Solvents.*—LC grade solvents, including glacial acetic acid, water, methanol, chloroform, and acetonitrile (J.T. Baker Inc.).

(**j**) *Lead oxide.*—Cat. No. 5727, ACS grade (Mallinckrodt, Inc., Science Products Div., St. Louis, MO).

(**k**) *Celite 545.*—Cat. No. C-212 (Fisher Scientific, Pittsburgh, PA).

(**l**) *Malachite green oxalate and leuco malachite green stock solutions.*—1 mg/mL in methanol. Prepare standard solutions of malachite green oxalate and leuco malachite green at concentrations of 0.50, 1.00, and 2.00 µg/mL fresh daily in mobile phase (85 + 15 methanol to aqueous acetate buffer) for LC retention time markers.

(**m**) *[Methano-^{14}C] malachite green chloride fortification solutions.*—Solutions of [Methano-^{14}C] malachite green chloride for fortifying samples consisted of 129.8 mg solute in 100 mL water for eggs and fry and 162.6 mg solute in 100 mL water for fry and adult muscle tissue. Prepare LC standards by dilution of 25 µL of each of the fortification solutions to 5 mL in 1% acetic acid (v/v) dissolved in acetonitrile.

(**n**) *Scintillation cocktails.*—Packard (Packard Instrument Co.) Permaflour V and Beckman (Beckman Instruments, Arlington Heights, IL) Ready Gel.

(**o**) *Carbosorb.*—Packard Instrument Co.

Test Specimens

(**a**) *Rainbow trout eggs.*—Obtained from Trout Lodge, McMillin, WA, as 1-day-old eggs.

(**b**) *Rainbow trout fry.*—Obtained from Trout Lodge, McMillin, WA, as 1-day-old eggs and hatched at the National Fisheries Research Center, La Crosse, WI.

(**c**) *Adult rainbow trout.*—Obtained from Trout Lodge, McMillin, WA, as 1-day-old eggs and hatched and raised at the National Fisheries Research Center, La Crosse, WI. Weights ranged from 1200 to 1500 g.

Sample Preparation and Extraction

Eggs and Fry

Homogenize samples of eggs and fry separately, approximating 0.5 g for each sample, with 4 mL 1% (v/v) acetic acid

in acetonitrile, on a Virtis Hi-speed "45" homogenizer for 1 min at 7700 rpm. Then, filter homogenate through Bakerbond filtration columns into 250 mL separatory funnels. Repeat the sample homogenization with 4 mL 1% (v/v) acetic acid in acetonitrile twice; then, rinse the homogenization cup 3 times with 2 mL 1% (v/v) acetic acid in acetonitrile into the Bakerbond column. Add 100 mL 1% anhydrous sodium bicarbonate to the eluate in the separatory funnel and partition by shaking 1 min with 10 mL chloroform. Repeat the partitioning procedure 2 times. Combine the chloroform partitions, and rotary evaporate at 30°C to dryness. Dissolve the resulting residue in 1.0 mL LC mobile phase, filter through an ACRO LC13 disposable filter, and analyze by the LC system.

Adult Muscle

Homogenize adult rainbow trout fillets by blending ca 1 in. pieces of frozen fillet with crushed dry ice in a Waring blender until pulverized to a fine powder, according to the method of Benville and Tindle (16). For each sample extraction, mix 5 g sublimated homogenate with 30 g anhydrous $NaSO_4$ in a 22 × 400 mm column and elute with 100 mL 1% (v/v) acetic acid in methanol by the method of Hesselberg and Johnson (17). Combine 50 mL eluate with 100 mL 1% anhydrous sodium bicarbonate in a 250 mL separatory funnel and partition by shaking 1 min with 10 mL chloroform. Repeat the partititioning step (i.e., the addition of 10 mL chloroform) 2 times. Combine the chloroform partition fractions and rotary evaporate at 30°C to dryness. Redissolve the residue with 7 1-mL portions of methanol, and then filter solution through an ACRO LC13 disposable filter. Concentrate the filtrate to 2.0 mL with nitrogen at room temperature and analyze by the LC system.

Recovery Study

Homogenates of eggs, fry, and adult muscle tissue of rainbow trout, prepared and extracted as previously described, were fortified with known amounts of [^{14}C] malachite green chloride. Egg samples weighing ca 0.5 g were fortified with either 40 or 80 µL of a 1:20 dilution of the [^{14}C] malachite green chloride fortification solution for eggs and fry in 1% (v/v) acetic acid in acetonitrile. Fry samples weighing ca 0.5 g were fortified with 40 µL of a 1:20 dilution of the same fortification solution. Homogenated muscle tissue samples weighing ca 5.0 g were fortified with 100 µL of a 1:100 dilution of the [^{14}C] malachite green chloride fortification solution for adult muscle tissue in 100 mL Methanol (see *Chemicals and Reagents* (1) for composition of fortification solutions). In addition to the LC method of analysis, three 1 mL subsamples of the extract eluate, as well as LC fractions collected every 30 s, were quantitated by liquid scintillation counting.

Exposure Study

Six adult rainbow trout held in pH 7.8 well water at 12°C were exposed to [^{14}C] malachite green chloride at a concentration of 1 mg/L for 1 h. After exposure, all test fish were rinsed and 3 were sampled immediately. The remaining 3 fish were transferred to fresh flowing well water and sampled after 5 days of withdrawal from the chemical. Two untreated fish of the same lot served as controls. Fillets with skins left on were removed, frozen, and homogenized with dry ice according to the procedure of Benville and Tindle (16). Subsamples of all fillet homogenates were processed by sample oxidation by scintillation counting and by our LC method with postcolumn sample oxidation to determine total malachite green residues

Table 1. Individual experimental measurements and mean amounts of labeled residues (with standard deviations in parentheses) as a percent of total [^{14}C] malachite green chloride (MGR) recovery and total residue recoveries as a percent of total [^{14}C] MGR fortification for extracts of rainbow trout eggs, fry, and muscle tissue

Sample type	Concentration of [^{14}C] MGR fortification, µg/g	Solution sample No.	Individual residues as a percent of total recovery						% Total [^{14}C] MGR recovered	Mean % of total [^{14}C] MGR recovered
			Unknown residue	Mean unknown residue	Leuco MGR residue	Mean leuco MGR residue	Chromatic MGR residue	Mean chromatic MGR residue		
Eggs	0.50	1	9	9	6	10	85	81	86	85
		2	7	(1.5)	17	(6.2)	76	(5.0)	85	(1.6)
		3	10		6		84		83	
Eggs	1.00	1	12	9	7	7	81	84	95	98
		2	10	(4.6)	7	(0.2)	82	(5.0)	99	(2.4)
		3	3		6		90		100	
Fry	0.65	1	11	13	10	11	79	76	66	68
		2	14	(1.8)	8	(3.6)	77	(4.4)	68	(2.2)
		3	13		15		71		71	
Muscle	1.00	1	0	0	85	89	15	11	63	66
		2	0	(0)	100	(10.5)	0	(10.5)	61	(4.5)
		3	0		100		0		71	
		4	0		75		25		72	
		5	0		70		20		68	
		6	0		94		6		63	

Table 2. Individual experimental measurements and mean concentrations (μg/g, with standard deviations in parentheses) of total [^{14}C] malachite green chloride (MGR) residues analyzed by the LC system in muscle tissue extracted from adult rainbow trout that were exposed to 1 mg/L [^{14}C] MGR in well water for 1 h[a]

Time that sample was taken after treatment	Fish number	Total concentration in tissue extracts, μg/g[b]	Mean total concentration in tissue extracts, μg/g	Individual residues as a percent of total concentration: Unknown residue	Mean unknown residue	Leuco MGR residue	Mean leuco MGR residue	Chromatic MGR residue	Mean chromatic MGR residue
0	1	0.8	1.0	25	23	45	47	29	30
			(0.15)		(2.4)		(2.4)		(4.0)
	2	1.1		24		49		26	
	3	1.0		21		45		34	
5 days	4	0.5	0.4	51	58	46	39	3.0	2.6
			(0.08)		(6.5)		(6.5)		(0.71)
	5	0.3		64		33		3.0	
	6	0.3		59		40		1.8	

[a] Individual residues are expressed as percents of the total LC concentration of MGR residues.
[b] The 0 h and 5 day samples analyzed by sample oxidation and scintillation counting contained 1.3 and 0.5 μg/g total [^{14}C] MGR residues, respectively.

present in the tissue. Individual fractions were collected at 30 s intervals during each LC injection and counted on the scintillation counter. The resulting counts were used to generate radiochromatograms.

Results and Discussion

Malachite green residues extracted from fortified homogenates of eggs, fry, and adult muscle tissue of rainbow trout (v/v) using 1% acetic acid in acetonitrile or in methanol were analyzed by the LC system with fractions collected every 30 s. Mean total recoveries from the fractions ranged from 66 to 98% when counted on a liquid scintillation counter, compared with the total radioactivity in standards. Some of the chromatic form of malachite green, however, was reduced to the leuco form in addition to an unidentified by-product during the procedures (Table 1).

Recoveries of total radioactive residue from eggs fortified with [^{14}C] malachite green chloride at concentrations of 0.50 and 1.00 μg/g were 85 and 98%, respectively. The mean composition of residues in the egg extracts fortified at a level of 0.50 μg/g was 81% chromatic malachite green, 10% leuco malachite green, and 9% an unknown peak eluting before the leuco malachite green peak. The mean composition of residues in the egg extracts fortified at a level of 1.00 μg/g was 84% chromatic malachite green, 7% leuco malachite green, and 9% the unknown peak. The mean total recovery in fry homogenates fortified at a level of 0.65 μg/g was 68%, and the composition of residues was 76% chromatic malachite green, 11% leuco malachite green, and 13% an unknown peak eluting before the leuco malachite green peak. Mean total recovery from adult muscle tissue homogenates fortified at a level of 1.00 μg/g was 66%, and the residue composition was 11% chromatic malachite green and 89% leuco malachite green.

Munns et al. (12) developed a specific method for analysis of leucogentian violet in chicken fat, and noted problems associated with the oxidation of leucogentian violet in gentian violet. Special precautions were recommended to avoid strong acids, bases, or certain metal ions (i.e., those in rust), which can catalyze the oxidation. In contrast, in the samples processed using our method, a 6 to 17% reduction of chromatic malachite green to the leuco form occurred for egg and fry samples, while a 75 to 100% reduction took place for adult muscle tissue samples. The unidentified peak, which eluted before the leuco malachite green peak, was found predominantly in the egg and fry samples and ranged from 3 to 14% of the total radioactivity recovered. No chromatic malachite green was reduced to the

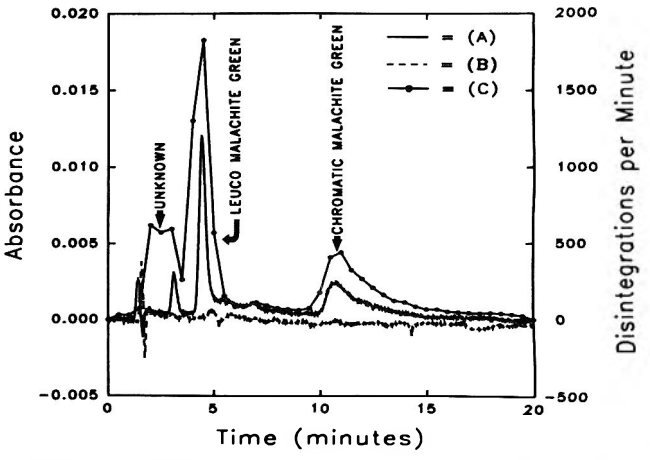

Figure 2. Representative LC chromatograms of muscle tissue extracts from adult rainbow trout exposed to a concentration of 1.00 mg/L of [^{14}C] malachite green chloride bath for 1 h and from an unexposed control. Spectrophotometric detection at 618 nm of (A) an exposed fish extract, (B) an unexposed fish extract, and (C) a radio-chromatogram (LC fractions collected at 30 s intervals and counted on a liquid scintillation counter) from an exposed fish extract.

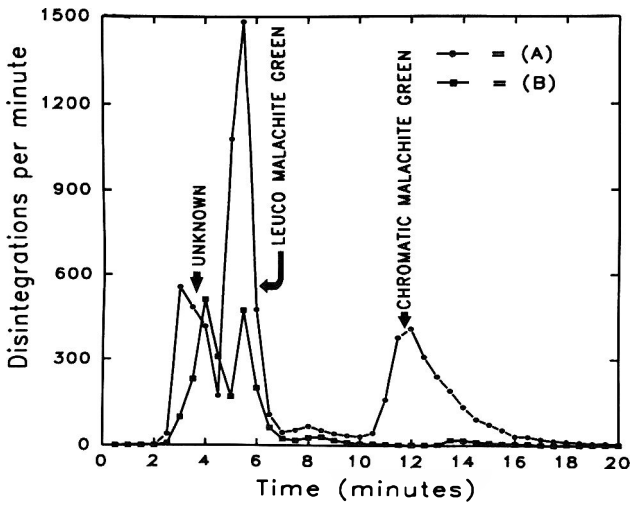

Figure 3. Representative LC radio-chromatograms of muscle tissue extracts from adult rainbow trout after (A) a 0 h and (B) a 5-day withdrawal from exposure to a concentration of 1.00 mg/L of [^{14}C] malachite green chloride bath for 1 h (LC fractions collected at 30 s intervals and counted on a liquid scintillation counter).

leuco form when a malachite green standard by itself (not in fish tissue) was processed using our method.

In our exposure study, we tested our method under operational conditions (Table 2). The samples taken immediately after exposure (0 h) contained 1.3 µg/g total radioactive malachite green residues as determined by sample oxidation and scintillation counting. The concentration of radioactive residues detected in LC fractions of muscle tissue extracts from samples taken after 0 h was 1.0 µg/g, 77% of the total found by sample oxidation and scintillation counting (Table 2). Chromatic malachite green constituted 30%, leuco malachite green 47%, and the unidentified peak 23% of the total residues (Figure 2). Samples taken 5 days after treatment was withdrawn contained 0.5 µg/g total radioactive malachite green residues by sample oxidation and scintillation counting. The mean concentration of radioactive residues detected by the LC system in tissue extracted from the 5 day samples was 0.4 µg/g, 80% of the total found by sample oxidation and scintillation counting. In these samples, chromatic malachite green constituted 2.6%, leuco malachite green 39%, and the unknown peak 58% of the total residues in tissue extracts by LC analysis (Figure 3).

Because leuco malachite green may be a predominant residue in fish samples, any method that is proposed should allow detection of this colorless form. Poe and Wilson (9) could detect malachite green residues only after fish tissue was frozen 6 weeks and the green color became visible. Possibly the leuco (colorless) form was oxidized to the chromatic form of malachite green during freezer storage. Bauer et al. (13) detected both chromatic malachite green and its leuco form by the LC system, but had to split the sample and oxidize one portion before LC injection (13). The amount of leuco malachite green was determined by the difference between the amount of chromatic malachite green found in the 2 sample portions.

Our method detects both leuco and chromatic forms of malachite green in a single LC injection using PbO_2 postcolumn oxidation. Although the susceptibility of malachite green to oxidation and reduction in fish tissues remains in problem, our method allows for rapid detection of both the chromatic and leuco forms of malachite green in fish tissue.

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Determination of Sulfamethazine in Bovine and Porcine Tissues by Reversed-Phase Liquid Chromatography

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A simple, sensitive, and rapid method for the liquid chromatographic determination of sulfamethazine in animal tissues was developed by using sulfaethoxypyridazine as the internal standard. Homogenized tissue is extracted with chloroform, and the sulfa drugs are back-extracted from chloroform into alkaline sodium chloride solution. The pH of the aqueous extract is adjusted to 6, and the sulfas are concentrated on a conditioned C_{18} cartridge and eluted with 1 mL methanol. Sulfamethazine and sulfaethoxypyridazine are separated from tissue co-extractives by reversed-phase chromatography on a C_{18} column by using 0.05M sodium dihydrogen phosphate–methanol (7 + 3). Detection is performed at 265 nm. The method has a detection limit of 2 ng/g. Results obtained by this method were compared with those obtained by the official thin-layer chromatography/densitometric method.

Sulfamethazine (SMZ), a highly effective, broad-spectrum antibacterial agent, is the most widely used sulfonamide in veterinary medicine for growth promotion and for prevention and treatment of bacterial infection in calves, foals, pigs, and cattle. Therefore, violative levels of SMZ residues might accumulate in animal tissues meant for human consumption. Moreover, SMZ is suspected to be a thyroid carcinogen (1). As a result, the use of SMZ in animal husbandry has rapidly gained nationwide attention in Canada. To protect the consumer and to ensure the marketability of Canadian pork and beef products, the Health Protection Branch of Health and Welfare Canada established in 1982 a tolerance level of 100 ng/g for SMZ residues in animal tissues (2).

Many chromatographic methods for the analysis of SMZ in animal tissues were reported, including thin-layer chromatography (TLC) (3–7), gas chromatography (GC) (8–11), liquid chromatography (LC) (7, 12–19), GC/mass spectrometry (MS) (9–11, 14, 19–23), and LC/MS (24). Most methods require elaborate preliminary cleanup procedures or have poor sensitivity and repeatability. When SMZ analysis is performed using nonspecific detectors (e.g., UV or electron capture) to support a regulatory action, such as the rejection and condemnation of an SMZ-contaminated carcass, a confirmatory test is usually required. The confirmatory test often uses detection techniques such as MS or infrared, because these techniques are capable of identifying the specific drug that exceeded the specified tolerance or action level. Reported methods that use LC techniques for SMZ cleanup or analysis with nonspecific detectors usually resort to chemical derivatization reactions to prepare volatile SMZ compounds suitable for GC/MS confirmatory analysis (14, 19). The probable reason is that the mass spectrometers owned by investigators using these methods are designed to accommodate only a GC/MS interface. In our laboratory, for example, SMZ is routinely analyzed using the TLC/densitometric method developed by Thomas et al. (3); confirmation of SMZ in tissue samples containing SMZ above the tolerance level is subsequently performed by using the official GC/MS method (23). Thus, 2 experiments are required to detect and confirm SMZ in animal tissues.

With the acquisition of an LC/MS interface in our laboratory, we tried to reduce to 1 the number of experiments required to determine and confirm SMZ in animal tissues. Thus, by developing a simple, rapid LC procedure with UV detection for the determination of SMZ in the low parts-per-billion range in animal tissues, we aimed to reduce the time required for SMZ analysis. Experimental conditions were selected to enable simultaneous determination and confirmation of SMZ in animal tissues by LC/UV and LC/MS, respectively, by a simple modification of the mobile phase composition. This paper describes the LC/UV method, which involves a solvent extraction step followed by a solid-phase extraction (SPE) cleanup and SMZ determination by reversed-phase LC.

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METHOD

Apparatus

(a) *Mechanical shaker.*—Eberbach flat bed (Eberbach Corporation, Ann Arbor, MI).

(b) *C_{18} SPE cartridges.*—3 mL (500 mg) Sep-Pak (Waters Chromatography Division, Mississauga, ON, Canada).

(c) *Centrifuge.*—MSE Coolspin 2 with fixed-angle rotors (Fisons, Sussex, England).

(d) *Homogenizer.*—Polytron with 20 mm probe (Brinkmann Instruments, Rexdale, ON, Canada).

(e) *Liquid chromatograph.*—Waters 600E System Controller, 610 Fluid Unit, 700 auto-injector (Waters Chromatography Division); Kratos 783 variable UV detector (Kratos Analytical, Ramsay, NJ); Spherisorb C_{18} ODS 5 μm, 250 × 4.6 mm id column (Phenomenex, Torrance, CA) (2).

(f) *Filters.*—Acrodisc® (Gelman Sciences, Montréal, PQ, Canada).

Reagents

(a) *Sulfamethazine (SMZ).*—Sigma Chemical Co., St. Louis, MO.

(b) *Sulfaethoxypyridazine (SEPD).*—American Cyanamid, Pearl River, LA.

(c) *Sodium dihydrogen orthophosphate, sodium chloride, and sodium hydroxide.*—BDH, Ltd, Toronto, ON, Canada.

(d) *LC/UV mobile phase.*—0.05M sodium dihydrogen phosphate–methanol (7 + 3, v/v).

(e) *Other reagents.*—All LC grade.

Preparation of Standard Solutions

(a) *SMZ and SEPD stock standard solutions.*—1000 μg/mL of each; prepare by accurately dissolving weighed amounts of each standard in methanol.

(b) *Standard working solutions.*—SMZ (10 μg/mL) and SEPD (20 μg/mL); prepare by appropriate dilution of each respective stock standard solution with water.

Sample Preparation

Accurately weigh ca 5.0 g homogenized blank (untreated) tissue extract into each of four 50 mL polypropylene tubes. Add 5, 25, 50, or 125 μL of the 10 μg/mL SMZ standard solution and 40 μL of the 20 μg/mL SEPD standard working solution to each tissue sample to yield samples with 10, 50, 100, and 250 ng/g SMZ, respectively, and a constant level (160 ng/g) of SEPD. For SMZ tissue samples, add 40 μL of 20 ng/mL SEPD standard to accurately weighed (5 g), homogenized samples.

Tissue Extraction

Add 25 mL chloroform to tissue samples containing internal standard, screw-cap the polypropylene tubes, and extract tissue on a mechanical shaker for 2 min. (*Note*: Operations involving chloroform must be conducted in the fume-hood. It is very important to adhere closely to the extraction time to maintain good sample recoveries.) Centrifuge the mixture at 3000 × *g* for 5 min. (*Note*: This step should be carried out in a centrifuge with fixed-angle rotors to enable easy transfer of the liquid phase after centrifugation.) Transfer the liquid phase into a 125 mL separatory funnel, and drain the lower layer into a 250 mL separatory funnel. Return the remaining upper aqueous layer in the 125 mL separatory funnel to the tissue plug in the polypropylene tube; repeat the extraction steps, centrifuge, and add the chloroform layer from the second extraction to the 250 mL separatory funnel containing chloroform from the first extraction.

Add 10 mL 10% sodium chloride (in 0.1M sodium hydroxide) to the 250 mL separatory funnel, stopper, and shake contents vigorously for 1 min. Let phases separate, transfer upper aqueous layer into a 50 mL polypropylene tube, and centrifuge at 1500 × *g* for 10 min. Pipet 8 mL of the upper aqueous layer into a 50 mL polypropylene tube containing 10 mL 1M sodium dihydrogen phosphate (pH = 6), and vortex-mix for 20 s.

Cleanup of Sample Extract on C_{18} SPE Cartridges

Load the sulfa extract onto a C_{18} cartridge previously conditioned with 20 mL methanol and 20 mL water. Cartridge must have a carbon loading content of 14% to obtain good recoveries. Rinse cartridge with 20 mL water, and elute sulfas immediately with 1 mL methanol. Do not allow the cartridge to dry before elution with methanol. Add 2 mL 0.05M sodium dihydrogen phosphate to the eluate, and filter through a 0.45 μm Acrodisc® filter into LC sample vials for analysis.

LC Analysis

Inject 20–50 μL filtered sample extract into LC system operated isocratically at a mobile phase flow rate of 0.8 mL/min. Measure peak heights of the chromatographic responses for SMZ and SEPD, and calculate the response ratio (peak height of SMZ/peak height of SEPD) for each spiked tissue sample. Plot a calibration curve of the response ratio versus the concentration of SMZ by using regression analysis. Also, calculate the response ratio for each incurred sample tested. Use the regression parameters for the standard curve to calculate the concentration, *X*, of SMZ in the samples by using the following equation:

$$X = \frac{(Y - b)}{m}$$

where *Y* is the response ratio for the sample and *b* and *m* are the intercept and slope, respectively, of the regression line.

Recovery, Inter- and Intra-assay Precision, and Accuracy

To determine the recovery of SMZ, standard solutions of SMZ at the same concentrations as those present in the SMZ-fortified tissue samples were injected into the LC system, and their UV responses were measured. The UV responses from SMZ-fortified tissue samples ($n = 4$ at each concentration) subjected to extraction-cleanup and LC were measured and compared with the UV responses from the standards. The accuracy of the method was assessed by measuring the response ratio of SMZ to SEPD in SMZ-fortified tissue samples and calculating the amounts of SMZ found in those samples by interpolation from the calibration curve. Intra-assay precision of the method was determined by replicate analyses ($n = 4$) of blank tissue samples fortified with SMZ at concentrations of 10, 50, 100, and 250 ng/g. In addition, tissue samples fortified with SMZ at concentrations of 20, 40, 80, and 120 ng/g and incurred porcine muscle and liver tissues were analyzed on 4 consecutive days to determine the day-to-day variation (interassay precision) of the method.

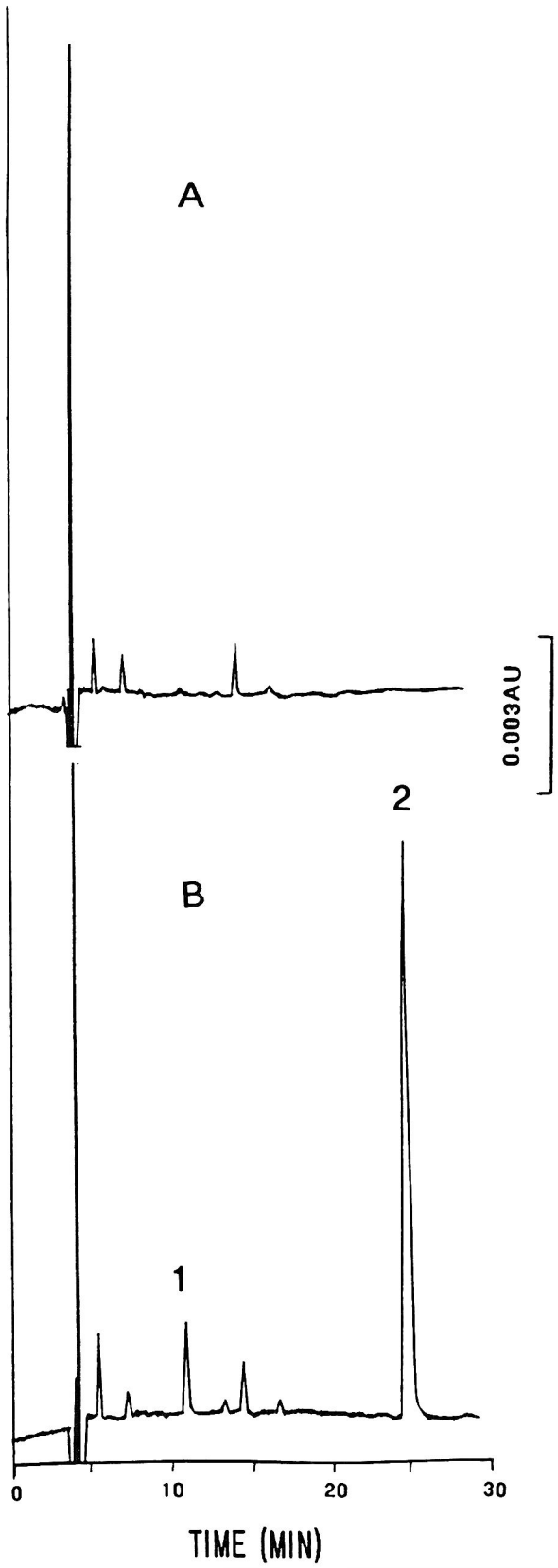

Figure 1. Typical chromatograms of a 20 μL injection of tissue extract (muscle) obtained from a control (untreated) pig (A) and a control porcine muscle sample fortified with SMZ and SEPD (internal standard) at concentrations of 10 and 160 ng/g, respectively, (B): 1, SMZ; 2, SEPD.

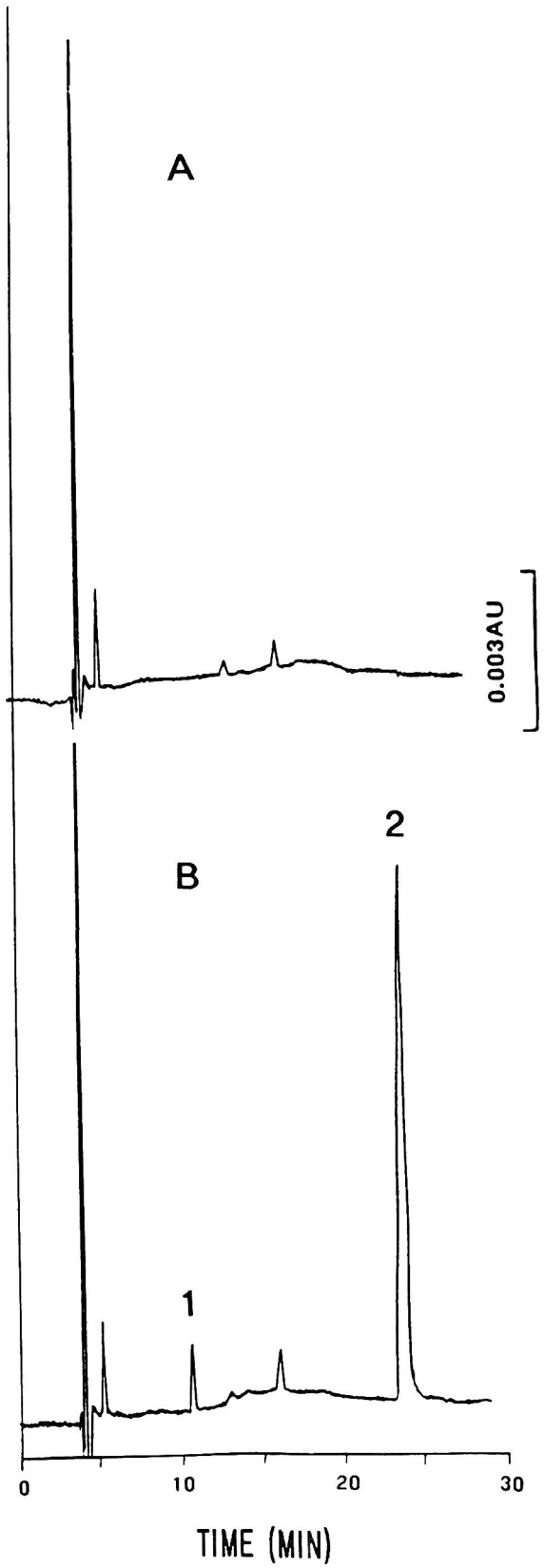

Figure 2. Typical chromatograms of a 20 μL injection of tissue extract (muscle) obtained from a control steer (A) and a control bovine muscle sample fortified with SMZ and SEPD (internal standard) at concentrations of 10 and 160 ng/g, respectively, (B): 1, SMZ; 2, SEPD.

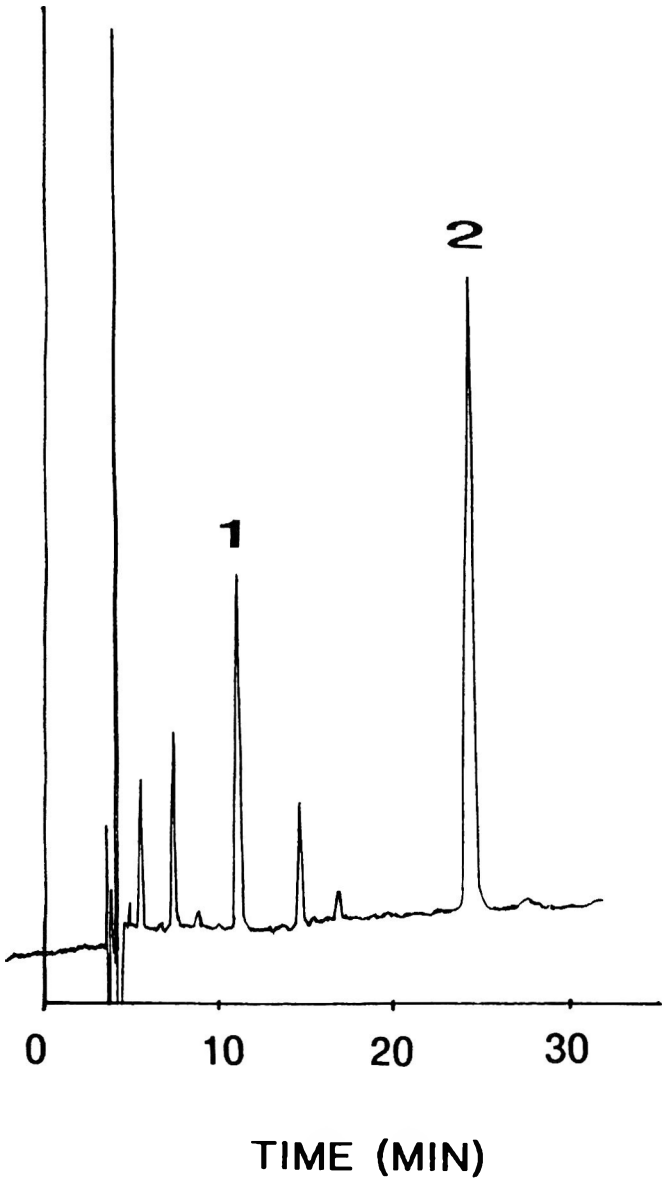

Figure 3. Chromatogram of a 20 μL injection of an SMZ-incurred liver extract (incurred liver A in Table 6), also fortified with 160 ng/g of SEPD as internal standard and found to contain 40.8 ng/g of SMZ: 1, SMZ; 2, SEPD.

Determination of SMZ in Interlaboratory Check Samples (Porcine Muscle)

Four sets of SMZ-incurred porcine muscle samples prepared for an interlaboratory check sample program for sulfa drugs were blind-analyzed for SMZ content.

Results and Discussion

Typical chromatograms of extracts obtained from control or SMZ- and SEPD-fortified porcine or bovine muscle are shown in Figures 1 and 2, respectively. Figure 3 shows a typical chromatogram of an SMZ-incurred liver extract. The retention times for SMZ and SEPD were 10.5 and 24.5 min, respectively (Figures 1B and 2B).

SEPD was selected as the internal standard because, in addition to having chromatographic properties similar to those of SMZ, SEPD was no longer being marketed by the manufacturer and was not expected to be found in animals intended for food (26). Additionally, SEPD was used to correct or compensate for any sample losses that may occur during the multistep procedure, especially because only a portion of the aqueous fraction was transferred for concentration and cleanup on the C_{18} cartridge. A concentration of 160 ng/g for the internal standard was selected, because at this concentration SEPD has about the same detector response as 100 ng/g SMZ (the tolerance or action level). This feature enables an analyst to immediately spot tissue samples that may be at or above tolerance levels and would be considered to be in violation during the chromatographic analysis. SMZ and SEPD were well resolved from each other, from other endogenous components, and from other frequently used sulfa drugs, including sulfadiazine, sulfathiazole, sulfamerazine, sulfanilamide, sulfamethoxypyridazine, sulfachloropyridazine, sulfamethoxazole, sulfadoxine, and sulfadimethoxine, with retention times of 3.8, 4.2, 6.4, 8.0, 11.7, 12.1, 14.0, 17.2, and 45 min, respectively. In addition, we found these other sulfa drugs to be efficiently extracted by our method. This finding, in our opinion, makes the method potentially useful for multiresidue sulfa drug analysis.

Average SMZ recoveries (± standard deviation [SD]) for SMZ-fortified porcine muscle, kidney, and liver tissues, respectively, were 92 ± 2, 86 ± 2, and 87 ± 1% (Table 1). The corresponding values for SMZ-fortified bovine muscle, kidney, and liver were, respectively, 93 ± 2, 95 ± 1, and 95 ± 1%. We obtained higher recoveries for SMZ-fortified bovine and porcine tissues than were generally obtained by SPE methods without prior solvent extraction; these higher recoveries may be attributable to the judicious combination of solvent extraction and C_{18} SPE cartridge cleanup.

Table 1. Recovery of SMZ from SMZ-fortified porcine tissue samples (n = 4)[a]

Tissue	SMZ added, ng/g tissue	Mean ± SD, ng SMZ/g: Standard	Mean ± SD, ng SMZ/g: Extracted sample	Recovery, %
Muscle	10.0	15 ± 2	13.8 ± 0.4	92
	50.0	76 ± 3	71.3 ± 3.3	94
	100.0	62 ± 2	56.8 ± 1.7	92
	250.0	158 ± 5	140.3 ± 11	89
Liver	10.0	15 ± 2	13.4 ± 0.8	89
	50.0	76 ± 3	65.5 ± 1.7	84
	100.0	62 ± 2	53.9 ± 1.2	86
	250.0	155 ± 8	133.5 ± 2.1	86
Kidney	10.0	15 ± 2	13.0 ± 1.4	86
	50.0	76 ± 3	69.4 ± 1.2	89
	100.0	61 ± 3	51.8 ± 2.5	87
	250.0	150 ± 7	130.3 ± 2.2	87

[a] Responses correspond to 50 μL injections of the 10 and 50 ng/g samples and 20 μL injections of the 100 and 250 ng/g samples.

Table 2. Recovery of SMZ from SMZ-fortified bovine tissue samples (n = 4)[a]

Tissue	SMZ added, ng/g tissue	Mean ± SD, ng SMZ/g: Standard	Mean ± SD, ng SMZ/g: Extracted sample	Recovery, %
Muscle	10.0	15 ± 2	14.1 ± 0.4	94
	50.0	76 ± 3	68.0 ± 0.9	91
	100.0	62 ± 2	57.1 ± 1.1	92
	250.0	158 ± 5	149.3 ± 2.5	96
Liver	10.0	15 ± 2	14.3 ± 0.6	95
	50.0	76 ± 3	70.5 ± 0.7	94
	100.0	62 ± 2	58.3 ± 0.6	94
	250.0	158 ± 5	148.9 ± 2.0	96
Kidney	10.0	15 ± 2	14.3 ± 0.4	95
	50.0	76 ± 3	70.9 ± 1.9	94
	100.0	62 ± 2	57.7 ± 2.3	93
	250.0	158 ± 5	149.0 ± 3.2	96

[a] Reponses correspond to 50 µL injections of the 10 and 50 ng/g samples and 20 µL injections of the 100 and 250 ng/g samples.

Table 4. Intra-assay precision and accuracy of LC method for the determination of SMZ-fortified blank porcine tissues (n = 4)

Tissue	SMZ added, ng/g tissue	Mean ± SD, ng/g	CV, %	Accuracy, %
Muscle	10.0	9.1 ± 0.2	2.3	–9
	50.0	49.1 ± 1.6	3.1	0
	100.0	101.5 ± 4.9	4.8	+2
	250.0	249.4 ± 10.5	4.2	0
Kidney	10.0	9.3 ± 0.8	8.4	–7
	50.0	48.9 ± 3.2	6.4	–2
	100.0	102.3 ± 2.8	2.7	+2
	250.0	249.4 ± 9.8	3.9	0
Liver	10.0	9.8 ± 0.6	5.6	–2
	50.0	50.4 ± 0.6	1.1	+1
	100.0	99.8 ± 0.9	0.9	0
	250.0	250.1 ± 4.2	1.7	0

Calibration curves (i.e., plots of the response ratio of SMZ to SEPD vs SMZ concentration) obtained for SMZ-spiked different tissue matrixes were linear and repeatable (Table 3). For tissue samples with SMZ concentrations greater than 250 ng/g, we recommend that either smaller sample sizes (1–4 g) be used or that SMZ concentrations be estimated from a calibration curve of response ratio vs SMZ concentration. The calibration curve should be obtained by using 200–1000 ng/g SMZ and 320 ng/g SEPD. The method accurately quantifies ≥ 5 ng/g SMZ residues in animal tissues. The detection limit (signal-to-noise ratio, 3) was 2 ng/g SMZ.

Tables 4 and 5, respectively, show the intra-assay accuracy and precision data for the LC determination of SMZ in porcine and bovine tissues. Average intra-assay variabilities of 3.6, 5.4, and 2.3% for porcine muscle, kidney, and liver, respectively (Table 4), and 3.8, 2.3, and 2.0% for bovine muscle, kidney, and liver (Table 5) were calculated. The tables show that the concentrations of SMZ added to animal tissue could be estimated at values ranging from –9 to +13% of the true values.

Results of the experiments conducted to determine the day-to-day (interassay) variation of the method are shown in Table 6. Average interassay variabilities of 1.9, 3.0, and 5.6% for bovine muscle, kidney, and liver, respectively, and 4.0, 4.3, and 2.3% for porcine muscle, kidney, and liver, respectively, were calculated.

Our current laboratory method for the determination of 20–200 ng/g SMZ in animal tissues (3) was also used to analyze the incurred tissue samples A and B. The results of our laboratory method were found to be slightly higher than those determined by LC (Table 7). The difference may be a reflection of the stability of SMZ even under frozen temperature conditions, because the LC analysis was performed about a month after the TLC analysis, during which time the samples were stored at –20°C.

The LC method was also used to analyze a set of SMZ-incurred tissues prepared for an interlaboratory check sample program for SMZ residues. The results of the LC analysis and the averaged results obtained by the TLC/densitometric method by 7 laboratories in the United States and Canada involved in the check sample program are presented in Table 7. Also included are the pretest results obtained at our laboratory before the check samples were shipped to the other laboratories. Except for sample S1139, for which the LC result is slightly higher, the results for samples S1136–S1138 compare

Table 3. Typical regression parameters for the analysis of SMZ in animal tissues

Tissue matrix	Regression equation[a]	Regression coefficient
Bovine muscle	$\bar{Y}_{muscle} = (0.0143 \pm 0.0005)X + (0.0129 \pm 0.0067)$	0.9999
Bovine kidney	$\bar{Y}_{kidney} = (0.0134 \pm 0.0004)X + (0.0095 \pm 0.0057)$	0.9997
Bovine liver	$\bar{Y}_{liver} = (0.0142 \pm 0.0006)X + (0.0098 \pm 0.0015)$	0.9998
Porcine muscle	$\bar{Y}_{muscle} = (0.0134 \pm 0.0006)X + (0.0113 \pm 0.0065)$	0.9998
Porcine kidney	$\bar{Y}_{kidney} = (0.0138 \pm 0.0009)X + (0.0303 \pm 0.0011)$	0.9991
Porcine liver	$\bar{Y}_{liver} = (0.0135 \pm 0.0004)X + (0.0079 \pm 0.0062)$	0.9999

[a] $\bar{Y}$ is the mean response ratio (UV detector reponse for SMZ/UV detector response for SEPD), and X is the concentration of SMZ, ng/g, added to blank tissue matrix.

Table 5. Intra-assay precision and accuracy of LC method for the determination of SMZ-fortified blank bovine tissues (n = 4)

Tissue	SMZ added, ng/g tissue	Mean ± SD, ng/g	CV, %	Accuracy, %
Muscle	10.0	11.3 ± 0.5	4.4	+13
	50.0	48.3 ± 2.6	5.4	−3
	100.0	99.8 ± 2.0	2.0	0
	250.0	250.4 ± 8.1	3.2	0
Kidney	10.0	10.9 ± 0.3	2.6	+9
	50.0	49.3 ± 0.6	1.3	−1
	100.0	99.3 ± 1.2	1.2	−1
	250.0	250.4 ± 10.5	4.2	0
Liver	10.0	10.6 ± 0.3	2.8	+6
	50.0	49.6 ± 0.7	1.3	−1
	100.0	99.3 ± 2.8	2.8	−1
	250.0	249.9 ± 2.2	0.9	0

Table 7. Comparison of SMZ concentrations in fortified porcine muscle and liver tissues, determined by LC and TLC/densitometry

Tissue	SMZ found, ng/g tissue		
	LC[a]	TLC[a,b]	TLC[c]
Porcine muscle A[d]	15.6 ± 1.3 (4)	20.5	—
Porcine muscle B[d]	181.7 ± 7.1 (4)	210.3	—
Porcine liver A[d]	40.5 ± 0.5 (4)	42.5	—
Porcine muscle S1136	122.1 ± 4.6 (2)	110.0 ± 0 (3)	119.0 ± 3.1
Porcine muscle S1137	77.2 ± 4.6 (2)	74.0 ± 2.9 (3)	79.6 ± 13.0
Porcine muscle S1138	74.3 ± 3.6 (2)	74.0 ± 5.0 (3)	66.3 ± 9.2
Porcine muscle S1139	72.8 ± 5.3 (2)	63.0 ± 1.5 (3)	66.6 ± 9.4

[a] Number of replicate analyses is in parentheses.
[b] Pretest results obtained by the TLC/densitometric method at the Health of Animals Laboratory, Saskatoon, Canada.
[c] Average results from the laboratories (n = 7) involved in the check sample program.
[d] Samples were stored at −20°C and analyzed by the LC method approximately 1 month after being analyzed by the TLC/densitometric method. Check samples S1136–S1139 were provided as part of an interlaboratory check sample program involving 7 regulatory laboratories in the United States and Canada and were analyzed by LC at about the same time that the samples were analyzed by the TLC/densitometric method.

favorably with both the pretest and interlaboratory results. Currently, it is possible to process and analyze 8 tissue samples for SMZ before the end of a working day. We have now modified the UV method to include the simultaneous detection of SMZ and sulfadimethoxine (SDM). We also replaced the phosphate buffer in our mobile phase with ammonium acetate buffer, which is a volatile matrix compatible with the thermospray LC/MS source conditions, to enable us to simultaneously determine and confirm SMZ and SDM residues in animal tissues by thermospray LC/MS (Boison et al., unpublished data).

Conclusion

A simple, sensitive, and rapid method for the LC determination of SMZ in animal tissues was developed. Test results for the determination of SMZ in animal tissues using the new method compare well with those obtained by the current AOAC method (3).

Table 6. Estimation of the interassay precision for SMZ determination in bovine and porcine tissues

Tissue	SMZ added, ng/g tissue	SMZ found, ng/g tissue				SMZ found, Mean ± SD, ng	CV, %
		Day 1	Day 2	Day 3	Day 4		
Porcine							
Muscle	40.0	41.0	40.1	41.0	41.2	40.8 ± 0.5	1.2
	80.0	78.4	83.0	80.1	80.2	80.4 ± 1.9	2.4
Kidney	20.0	22.5	19.3	19.4	20.4	20.4 ± 1.5	7.4
	120.0	119.6	118.2	115.9	118.3	118.0 ± 1.5	1.3
Liver	20.0	20.2	20.9	20.4	20.5	20.5 ± 0.3	1.5
	120.0	120.3	123.6	117.0	121.3	120.6 ± 2.7	2.2
Bovine							
Muscle	40.0	40.2	39.7	39.3	41.0	40.1 ± 1.8	4.5
	80.0	79.8	80.7	83.5	80.7	81.2 ± 1.6	2.0
Kidney	20.0	20.2	21.6	22.4	20.4	21.2 ± 1.0	4.7
	120.0	118.7	118.2	117.0	120.2	118.5 ± 1.3	1.1
Liver	20.0	20.8	20.3	20.3	17.5	19.7 ± 1.5	7.6
	120.0	126.2	118.8	116.0	120.1	120.3 ± 4.3	3.6
Incurred muscle A		15.3	15.6	17.4	14.2	15.6 ± 1.3	8.3
Incurred muscle B		175.4	182.0	178.0	191.5	181.7 ± 7.1	3.9
Incurred liver A		40.8	40.0	40.0	40.9	40.4 ± 0.5	1.2

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DRUGS, COSMETICS, FORENSIC SCIENCES

Analysis of Penicillin G in Milk by Liquid Chromatography

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A liquid chromatographic (LC) method that was previously developed for penicillin G residues in animal tissues has been adapted to milk and milk products. After protein precipitation with sodium tungstate, samples are applied to a C_{18} solid-phase extraction cartridge, from which penicillin is eluted, derivatized with 1,2,4-triazole–mercuric chloride solution, and analyzed by isocratic liquid chromatography (LC) on a C_{18} column with UV detection at 325 nm. Quantitation is done with reference to penicillin V as an internal standard. Penicillin G recoveries were determined to be >70% on standards fortified at 3–60 ppb. Accuracy approached 100% using the penicillin V internal standard. The detection limit for penicillin G residues was 3 ppb in fluid milk. Samples may be confirmed by thermospray/LC at concentrations approaching the detection limit of the UV method.

Procaine penicillin G (Pen G), alone or in combination with dihydrostreptomycin sulfate (DHS), is commonly used in the treatment of cattle for mastitis, shipping fever, and other infections that respond to these antibiotics. In the United States, 17 drug formulations containing Pen G, 6 of which also contain DHS, are approved for veterinary use in dairy cattle (1). Fourteen such Pen G formulations are approved in Canada, 6 of which include DHS. After the final treatment of dairy cattle with the label dose, their milk must be discarded for 72 h (96 h after treatment with mixed formulations containing DHS) (2). A similar situation exists in the United States, where required discard times vary from 24 to 96 h, depending on the product and method of treatment (1). Other penicillin formulations are available, some of which contain the longer-acting benzathine Pen G, but these formulations are not approved for use in dairy cattle. Thus, in addition to the risk of Pen G residue contamination in milk resulting from incorrect use of approved products, the use of unapproved products can also cause residue accumulations. Such Pen G residues, apart from the allergic reactions they may elicit in hypersensitive individuals, may also inhibit starter cultures used in the production of yogurt, cheese, and other milk products.

Various test kit technologies are available to screen fresh milk samples for antibiotic residues. In general, these test kits offer good sensitivity and should be suitable for detecting Pen G at the recommended (3) maximum residue limit of 4 ppb in milk. However, improvement in the sensitivity of laboratory methods for the confirmation of penicillin residues is needed (3).

Several chromatographic procedures for the analysis of penicillin residues were reported in recent years. Terada and Sakabe (4) used solid-phase extraction (SPE) cartridges to clean up milk samples containing Pen G, penicillin V (Pen V), and ampicillin prior to liquid chromatographic (LC) analysis on a C_{18} column and detection at 210 nm using a UV detector. Recoveries were approximately 90%, and detection limits were 30 ppb. Munns et al. (5) reported a multiresidue method for the determination of 8 penicillins in milk by using LC analysis with fluorescence detection. Recoveries were determined from spikes of 25–100 ppb penicillins. Wiese and Martin (6) used solvent extraction to recover and concentrate penicillin residues from milk before derivatizing the penicillin with mercuric chloride in the presence of imidazole to form the mercuric mercaptide of the penicillenic acid. These researchers used LC on a C_{18} analytical column and applied a technique that used digital subtraction of a blank sample on a data system to improve sensitivity and obtain a detection limit of about 2 ppb. An LC method using ultrafiltration of the sample, diode array detection, and mass spectrometric (MS) confirmation was reported by Tyczkowska et al. (7). This technique had detection limits of 10 ppb for the UV method and 100 ppb for the LC/MS method.

More recently, Moats and Malisch (8) determined cloxacillin and Pen V in milk at 1 ppb by using an automated LC cleanup procedure previously applied to ampicillin (9). Fletouris et al. (10) analyzed some monobasic penicillins (Pen G, Pen V, oxacillin, and cloxacillin) in milk at 3–4 ppb by using an ion-pair LC method. The use of capillary gas chromatography for the determination of Pen G and a number of other penicillins in milk was reported by Meetschen and Petz (11), and they achieved detection limits of less than 1 ppb.

Methods reported to date for penicillin involve various degrees of difficulty and time requirements, but the more sensitive methods have features that may be less than ideal for use

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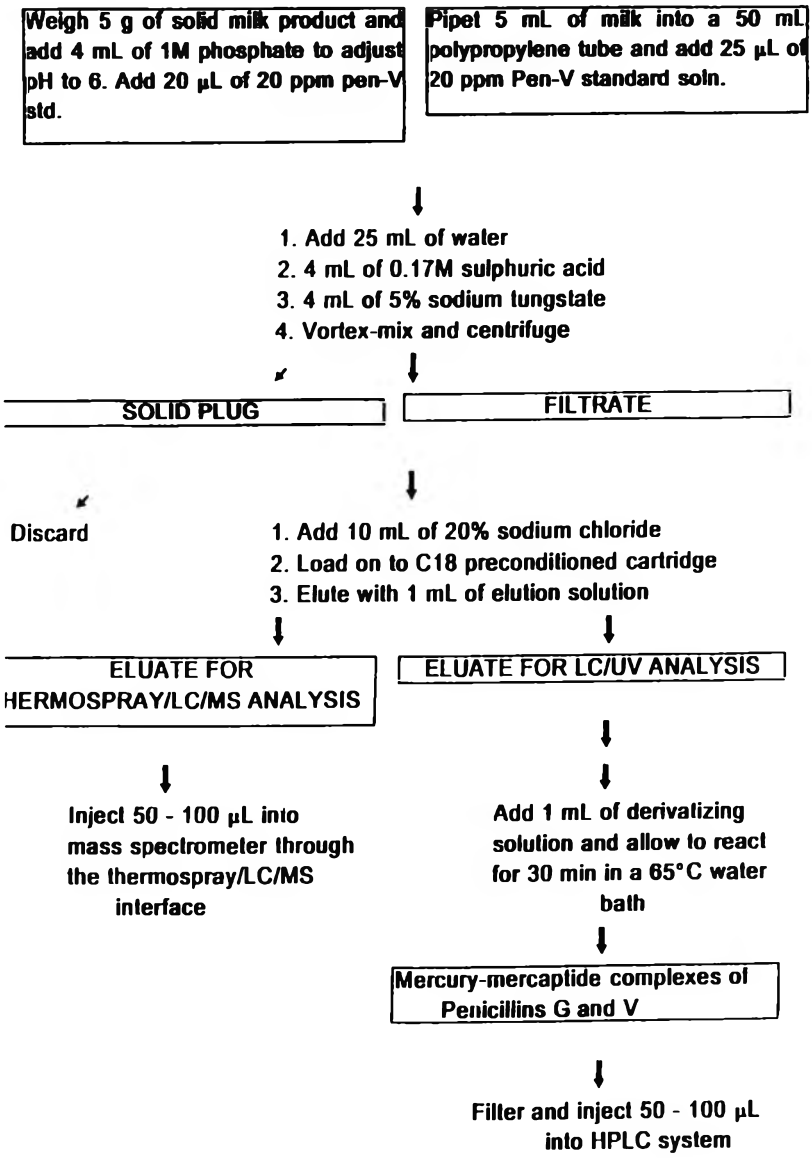

Figure 1. Flowchart of extraction/cleanup procedure for the analysis of penicillin G in milk and milk products.

in a routine regulatory laboratory. Therefore, we decided to investigate the application of a rapid method, which was recently developed in our laboratory for the analysis of Pen G (12) in animal tissues, to the analysis of Pen G residues in fluid milk. We also demonstrated that, with a slight modification of the milk protocol, solid milk products, yogurt, and cottage cheese fortified with Pen G can be successfully analyzed.

Materials and Methods

Reagents

(a) *Acetonitrile.*—LC grade (absorbance <0.02 at 210 nm and higher wavelengths).

(b) *Water.*—Obtained from a Barnstead Reverse Osmosis/Nanopure ultrafiltration unit.

(c) *Derivatizing reagent for penicillin.*—2M 1,2,4-triazole containing 0.001M mercuric chloride. Weigh 34.45 g 1,2,4-triazole (Aldrich, Milwaukee, WI) into a 400 mL beaker, add 150 mL water, and stir with a magnetic spinbar to dissolve. Add 25 mL 0.01M $HgCl_2$ solution, mix, and adjust to pH 9.0 ± 0.5 with 5M NaOH. Transfer quantitatively into a 250 mL volumetric flask and dilute to volume with water.

(d) *Procaine Pen G and Pen V (phenoxymethyl penicillinic acid) standards.*—Sigma Chemical Co., St. Louis, MO.

(e) *Phosphate buffer for penicillin analysis.*—0.2M. Weigh 0.994 g dibasic sodium phosphate (anhydrous) and 1.794 g monobasic sodium phosphate (monohydrate), dissolve in 80 mL water, and dilute to 100 mL in a volumetric flask (pH = 6.5).

(f) *Elution solution for LC/UV.*—Combine 60 mL acetonitrile and 5 mL 0.2M phosphate buffer in a 100 mL volumetric flask, and dilute to volume.

(g) *Elution solution for LC/MS.*—Combine 60 mL acetonitrile with 5 mL 0.2M ammonium acetate solution in a 100 mL volumetric flask, and dilute to volume.

Table 1. Interassay precision and accuracy for the determination of penicillin G in fluid milk

Fortification, ng/mL	Mean conc. found ± SD, ng/mL	Accuracy, %	CV, %
6.0 (7)[a]	6.2 ± 0.2	103	3
50.0 (7)	49.1 ± 1.4	98	3

[a] Numbers in parentheses represent the number of replicate analyses conducted at each fortification level.

(h) *Phosphate buffer.*—0.1M containing 0.0157M thiosulfate. Weigh 4.696 g dibasic sodium phosphate (anhydrous), 8.969 g monobasic sodium phosphate (monohydrate), and 2.482 g anhydrous sodium thiosulfate. Dissolve in 800 mL water and dilute to volume in 1 L volumetric flask.

(i) *Mobile phase for LC.*—Measure 750 mL 0.1M phosphate buffer containing 0.0157M thiosulfate into a 1 L volumetric flask, dilute to volume with acetonitrile, mix thoroughly, and filter through a 0.45 µm unit under vacuum.

(j) *Mobile phase for LC/MS.—Solvent A.*—Filter 1 L acetonitrile through a 0.45 µm unit under vacuum. *Solvent B.*—Measure 900 mL 0.15M ammonium acetate into a 1 L volumetric flask, dilute to volume with acetonitrile, mix thoroughly, and filter through a 0.45 µm unit under vacuum.

Apparatus

(a) *SPE cartridges.*—6 mL. (500 mg) BondElut C_{18} (Varian, Harbor City, CA).

(b) *Vortex mixer.*—Variable speed mixer (Canlab Division, Baxter Diagnostics Corp., Mississauga, ON, Canada).

(c) *SPE vacuum manifold.*—Spe-ed Mate™-30 vacuum manifold (Applied Separations, Bethlehem, PA).

(d) *LC/UV equipment.*—Waters 610 fluid unit with 600E system controller, 700 satellite WISP, 486 UV-VIS detector, Nova-Pak C_{18} column, 4 µm packing, 15 cm × 3.9 mm id (Waters Canada Ltd., Mississauga, ON, Canada); ABB SE120 strip-chart recorder (Fisher Scientific, Nepean, ON, Canada).

(e) *LC/MS equipment for confirmatory analysis.*—Trio II mass spectrometer with thermospray/plasmaspray interface (VG Instruments [Canada] Inc., Pointe-Claire, QUE, Canada), 600MS solvent delivery system (Waters Canada Ltd.), and Rheodyne 7125 sample injection valve. LC column as described above.

Table 2. Recovery and intra-assay precision for the determination of penicillin G in fluid milk

Fortification, ng/mL	Mean conc. found ± SD, ng/mL	Recovery, %	CV, %	Accuracy, %
3.0 (4)[a]	3.46 ± 0.04	70 ± 8	1.2	115
10.0 (4)	9.68 ± 0.25	71 ± 3	2.6	97
20.0 (4)	19.5 ± 0.43	77 ± 3	2.2	98
30.0 (4)	29.8 ± 0.78	88 ± 8	2.6	99
60.0 (4)	60.0 ± 2.9	81 ± 5	4.8	101

[a] Numbers in parentheses represent the number of replicate analyses conducted at each fortification level.

Determination

Preparation of standards.—(*1*) Pen G stock standard (1000 ppm): Dissolve 0.170 g procaine Pen G in 100 mL methanol–water (1 + 1, v/v). Prepare 1 ppm and 10 ppm working standards by serial dilution. (*2*) Pen V stock standard (1000 ppm): Dissolve 0.111 g phenoxymethyl penicillinic acid in 100 mL water. Prepare 20 ppm working standard by serial dilution.

Preparation of calibration curve and addition of internal standard.—Pipet 5 mL milk into a 50 mL polypropylene tube, and fortify with 20 µL of a 20 ppm Pen V standard solution (internal standard). To construct a calibration curve, pipet 5 mL

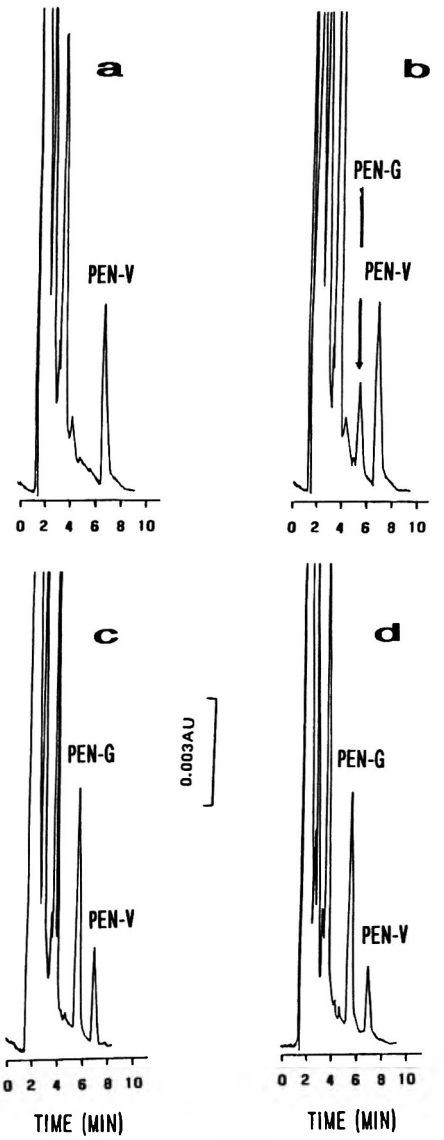

Figure 2. Chromatograms of the following extracts: control milk with Pen V internal standard (a), bulk tank milk fortified with Pen G (b), cottage cheese fortified with Pen G (c), and yogurt fortified with Pen G (d) (Figures b–d also contain Pen V internal standard).

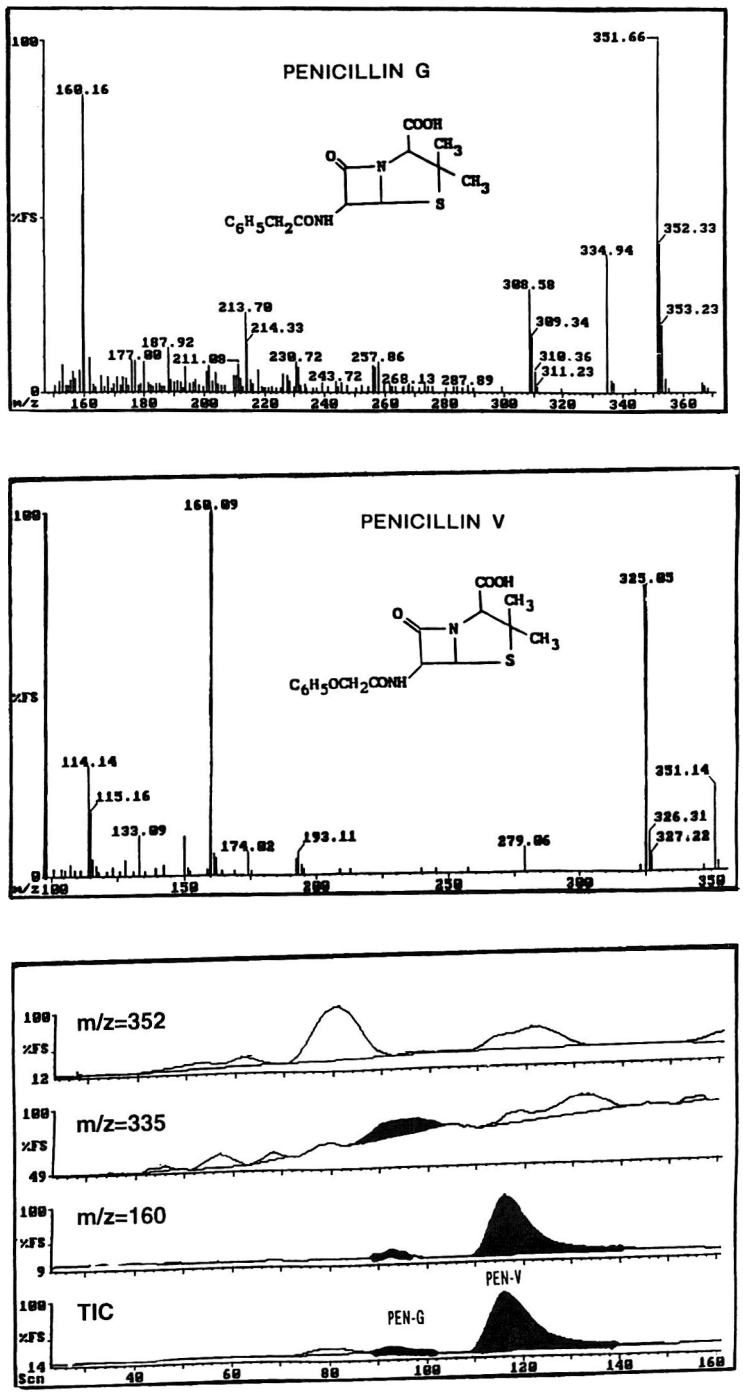

Figure 3. Thermospray/mass spectral analysis of bulk tank milk fortified with 5 ppb penicillin G and 80 ppb penicillin V internal standard; TIC = total ion chromatogram.

of blank (unfortified control) milk into each of 5 polypropylene tubes, and add 20 µL of a 20 ppm Pen V standard to each sample. Add 15, 50, and 100 µL of a 1 ppm Pen G standard and 15 and 30 µL of a 10 ppm Pen G standard, respectively, to each of the 5 samples to give milk standards containing penicillin equivalencies of 3, 10, 20, 30, and 60 ng/mL, together with a constant amount (80 ng/mL) of Pen V.

Sample extraction.—Add 25 mL water to each sample, then add 4 mL 0.17M H_2SO_4 and 4 mL of a 5% (wt/v) sodium tungstate solution. Vortex-mix for 30, centrifuge 10 min at 1500 × *g*, and filter the supernatant into a clean 50 mL polypropylene tube. Add 10 mL of a 20% sodium chloride solution to the contents of the tube, and vortex-mix 10 s. Load each sample onto a C_{18} SPE cartridge that was previously conditioned with 20 mL methanol, 20 mL water, and 10 mL of a 2% sodium chloride solution. Rinse each cartridge with 10 mL of a 2% sodium chloride solution and 10 mL water. Elute penicillins with 1 mL of elution solution into a 15 mL glass centrifuge tube (*Note*: Samples intended for LC/MS confirmation should be eluted with 750 µL ammonium acetate–acetonitrile elution so-

lution instead of the phosphate buffer–acetonitrile solution.) For samples intended for LC/UV, add 1 mL of derivatizing reagent to the eluate, cap the tube with a Teflon-lined screwcap, vortex-mix 10 s, and place the sample in a 65°C water bath for 30 min. Remove sample from water bath, and allow to cool to room temperature in a beaker of water.

LC determination.—Filter sample through 0.45 μm Acro disc filter, and inject a 50–100 μL aliquot into the LC system by using the mobile phase (25% acetonitrile–75% phosphate buffer, pH 6.5) at a flow rate of 0.8 mL/min.

LC/MS confirmation.—Filter sample as above. Inject a 50–100 μL aliquot using a gradient mobile phase of solvents A and B (*see Reagents*, [j]) as follows: 0–10 min, 100%B; 10–20 min, 70%B–30%A; 20–30 min, 100%B; flow rate, 09 mL/min. The capillary probe tip and source of the VG Trio II mass spectrometer were operated at 255 and 180°C, respectively.

Precision, accuracy and recovery.—Interassay precision of the method was determined by duplicate assays on 4 successive days of blank (control) milk samples fortified with Pen G at 6 and 50 ng/mL. Intraassay precision of the method was determined by replicate analyses ($n = 4$) of blank (control) milk samples fortified with Pen G at various concentrations. The accuracy of the method was evaluated by calculating (from the calibration curve) the amounts of Pen G found in blind-fortified samples. To determine procedure recoveries, the UV responses for Pen G in fortified samples subjected to extraction, cleanup, and LC analysis were compared with those of equivalent external Pen G standards.

Application.—The method was applied to the analysis of Pen G in fresh bulk milk, fat-reduced homogenized milk (1 and 2% fat, respectively), and homogenized whole milk. Blank (control) bulk tank milk was spiked at 20 and 100 ppb with Pen G. Samples were divided into 5 groups, each containing samples at both fortification levels. One group was analyzed immediately. The remaining 4 groups were refrigerated at 4°C and analyzed after 1, 2, 3, and 6 days of storage (one sample each day).

Results and Discussion

The method (Figure 1) is simple, sensitive, and reproducible. An analyst can prepare 8–12 samples for LC analysis in 3–4 h; therefore, LC results are available the same day. The interassay precision and the accuracy, determined at 2 levels of fortification in milk (Table 1), were better than those previously reported for tissue (11). Intraassay precision was also excellent, and recoveries were >70% (Table 2).

Also shown in Figure 1 is a modification of the milk protocol that included an initial adjustment of the solid milk pH before sample treatment, as was done for fluid milk samples. With this modification, yogurt and cottage cheese fortified with Pen G at 20 and 100 ppb levels could be recovered with ≥80% efficiency; recovery efficiencies for solid milk products without pH adjustment were about 60%. Figure 2 illustrates the chromatographic separation after extraction and cleanup achieved for Pen G in milk at 10 ng/mL (Figure 2b) and in cottage cheese (Figure 2c) and yogurt (Figure 2d) at 100 ng/g. Each sample contained 80 ng/g Pen V as internal standard. The detection of Pen G in milk at 5 ng/mL by using thermospray/LC/MS is shown in Figure 3. By using selected ion monitoring, bulk tank milk fortified with 5 ppb Pen G gave detectable ion fragments at *m/z* 335 and 160. The third ion fragment used in monitoring for Pen G, *m/z* 352, is less intense and was not detected at this concentration.

Table 3. Refrigeration (4°C) effect on Pen G stability in milk

Storage, days	Pen G found in fortified samples, ppb[a]	
	20.0 ppb spike	102.5 ppb spike
0	21.0	102.5
1	21.0	102.6
2	21.0	96.0
3	20.0	98.0
6	19.0	98.0

[a] Duplicate analyses were conducted at each level of fortification.

Milk samples fortified with 20 and 100 ppb Pen G remained stable for 6 days under normal refrigeration (Table 3). This result was in agreement with findings previously reported (6).

The method permits quantitative determination of Pen G residues at ≥3 ng/mL in milk using LC. Calibration curves plotted from the response ratios (Y) of Pen G/Pen V were linear between concentrations (X) of 3 and 60 ppb. The mean regression equation was as follows:

$$\bar{Y} = [0.01954 \pm 0.001717]\bar{X} + [0.01348 \pm 0.01478]$$

with a correlation coefficient of $r = 0.9996 \pm 0.0005$, from which a detection limit of 3 ppb was calculated. In addition, thermospray/LC/MS confirmation can be performed at levels approaching the tolerance level for Pen G in milk. The quantitative methodology appears amenable to further automation and should be suitable for use in a regulatory program.

Acknowledgment

We extend our thanks to R. Gedir for his skilled technical assistance in conducting the thermospray/LC/MS analyses.

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DRUGS, COSMETICS, FORENSIC SCIENCES

International Validation Study for the Determination of Chloramphenicol in Bovine Muscle

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A gas chromatographic (GC) procedure for the quantitation and GC/negative ion chemical ionization mass spectrometric (NICIMS) confirmation of chloramphenicol in calf muscle tissue was the subject of a validation study. Five analysts representing 5 laboratories in 4 countries participated in the quantitative method and analyzed 7 randomly numbered blind triplicates at 4 fortified and 3 incurred tissue concentrations on 3 separate days. The chloramphenicol concentrations ranged from 0 to 2.5 ppb. All data were reported to 3 significant figures. The coefficients of variation were 9.5–28.7% for repeatability and 14.6–38% over the study range for reproducibility. NICIMS data representing 3 laboratories in 3 countries successfully confirmed chloramphenicol in samples at 0.6 ppb or greater with no false positives in blank tissues.

Chloramphenicol (CAP) has a broad spectrum of activity against gram-positive and gram-negative bacteria and rickettsiae. Residues of CAP in edible tissue are a public health concern because CAP can cause aplastic anemia in humans (1) and because use of CAP in dairy cows has been linked to CAP-resistant *Salmonella* infections in humans (2). Therefore, CAP is not approved for use in animals used as food in the United States (3). Because of the varying regulatory requirements on the use of CAP in different countries, an acceptable, internationally sanctioned method capable of detecting and confirming CAP at <1 ppb was needed.

CAP has been studied exhaustively, and many different analytical approaches have been developed. Reviews of CAP chromatographic methods have been published (4, 5). Prior to the development of this method, the U.S. Department of Agriculture (USDA), Food Safety and Inspection Service (FSIS), had been screening cattle and swine tissues for CAP by using a qualitative enzyme immunoassay card test (EZ-SCREEN QUICK-CARD test kit 205-22, Environmental Diagnostics, Inc., Burlington, NC), but a confirmation test for possible presumptive positives from the card test was not available. An earlier FSIS gas chromatography/electron capture (GC/EC) quantitation test (6) with negative ion chemical ionization mass spectrometry (NICIMS) confirmation (7) had a detection limit of 10 ppb in muscle tissue for bovine, swine, and poultry but was not considered sensitive enough. In the present work, the earlier method was extended by adding ß-glucuronidase enzyme hydrolysis to free CAP from its glucuronide and the liquid–liquid and the solid-phase extractions (SPE) of Johannes et al. (8). This method (8) was summarized in English by both the

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J. T. Baker Chemical Company (9) and by Allen (4). Although the CAP-3-glucuronide metabolite is probably not present in calf muscle, enzyme hydrolysis was retained and evaluated, because this method was to be extended to analysis of swine muscle. The presence of CAP glucuronide was indicated in a swine muscle sample (10). Instead of using the mono-CAP analogue of CAP as the internal standard, as in the original USDA method, the *meta* isomer of CAP was substituted. *m*-CAP was first used as an internal standard in a GC/EC method by Arnold and Somogyi (11).

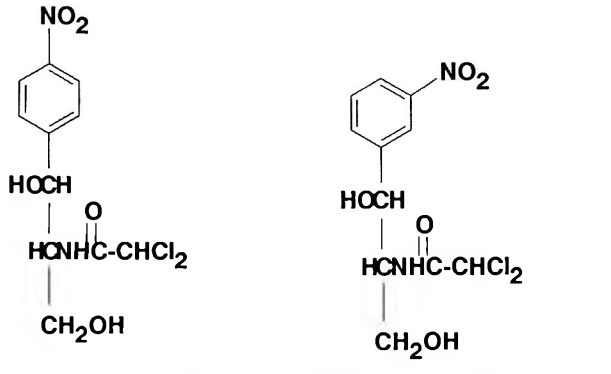
Validation Protocol

Study Design

Five laboratories from 4 countries participated: 1 each from the United States, Canada, and Taiwan and 2 from Australia. Calf muscle samples were randomly numbered within each of 3 sets of 4 fortified and 3 incurred samples of 0–2.5 ppb CAP. Samples were analyzed on 3 separate days (1 set per day) for a total of 21 samples. Three laboratories (United States, Canada, and Australia) were requested to confirm the presence or absence of CAP in selected incurred samples at all 3 target concentrations of 0.5, 1.5, and 2.5 ppb; in 0.6 ppb fortified tissue; and in selected blank tissues. No distinction was made between fortified and incurred samples. On each day an analyst analyzed a set of 7 samples along with 3 samples fortified at 0.5, 1.0, and 2.0 ppb to construct a calibration curve for quantifying CAP residues. To each of the 10 samples (controls plus study samples), *m*-CAP at 1 ppb was added as surrogate analyte and processed through the method for internal quality control. For positive results, the peak area or peak height ratio (peak height of CAP to peak height of *m*-CAP) was used in the calculations. The structures of CAP and *m*-CAP are shown in Figure 1.

Prepared CAP–methanol mixtures in sealed ampoules were provided. Samples were fortified by addition of 100 µL of the mixture to 10 g ground muscle. For incurred or blank tissue matrix, only 100 µL methanol was added. All analytical values were reported to the nearest 0.01 ppb.

Confirmation was performed on designated samples by 3 participating laboratories using the procedure and criteria stated in the method below. Results were reported as confirmed (C) or not confirmed (NC).



CHLORAMPHENICOL METACHLORAMPHENICOL

Figure 1. Chemical structures.

Incurred Tissues

Incurred calf muscle tissues were obtained by using intravenous dosing protocols developed for use in calves (12). Concurrent dosing was done in the United States and Australia. In the United States, the calves were dosed by the Agricultural Research Service in College Station, TX; in Australia, dosing was done by the Australian Government Laboratories in New South Wales. In each study, a control calf was not medicated.

Three calves (2 in the United States and 1 in Australia), which weighed ca 90 kg each, were dosed intravenously with a 150 mg/mL CAP–propylene glycol solution twice at 24 h intervals, for a total dose of 66 mg/kg. The calves were slaughtered at different time intervals to produce the target CAP concentrations in the muscle tissues. The U.S. calves were slaughtered in an approved abattoir 64 and 68 h after dosing. The 68 h calf had a CAP concentration of ca 2.5 ppb. FSIS blended some of the 64 h calf muscle with blank calf muscle to achieve a second incurred tissue at ca 1.5 ppb. Similarly, the Australian calf was slaughtered at 66 h after the last CAP dosing, producing an approximate concentration of 0.5 ppb. Approximately 10 kg of muscle tissue was collected from each medicated and control calf, frozen at −20°C, and shipped to a designated laboratory for analysis to ensure that the target concentrations were achieved. The calf muscle tissues were then prepared and homogenized for use in the validation study by using a Hobart Model 841810 grinder fitted with a plate having 0.20 cm holes.

Experimental

Theory

This procedure is based on extracting CAP from 10 g muscle by using β-glucuronidase to hydrolyze any monoglucuronide and adding *m*-CAP as a surrogate analyte. Free CAP is then extracted with ethyl acetate. The CAP is partitioned into a 4% NaCl solution, purged with nitrogen, and isolated further by SPE with a C_{18} SPE cartridge. The cartridge is washed with methanol–water (20 + 80) and then eluted with acetonitrile. The eluate is evaporated to dryness and silylated. CAP is determined by GC/EC detection and confirmed on an OV-1 capillary column by GC–NICIMS.

Apparatus

(a) *Vacuum manifold device.*—J. T. Baker or Analytichem, for aid in washing and elution of C_{18} cartridges.

(b) *N-Evap.*—Organomation Associates, or equivalent.

(c) *Pipettes.*—Glass serological (10 mL) and Eppendorf (50–200 µL).

(d) *Test tube racks.*

(e) *Centrifuge tubes.*—Glass, round bottom, 50 mL Corex, with Teflon-lined screw caps (Corning No. 85422A, or equivalent).

(f) *Centrifuge.*—Damon IEC Division Model PR-7000 with IEC No. 253 rotor; cup size, IEC catalog No. 320, or equivalent.

(g) *Syringes, microliter.*—Hamilton No. 701 or No. 1701, 10 μL.

(h) *Vortex mixer.*—Labline Supermixer Model No. 1290, or equivalent.

(i) *Homogenizer.*—Ultra Turrax Model SDT with microshaft.

(j) *Incubator, low temperature.*—Precision Scientific Freas Model 825, or equivalent.

(k) *Heating module.*—Reacti-Therm, Pierce Model No. 18780, or equivalent.

(l) *Vials, autosampler, 1 mL, conical.*—Chemical Research Supplies, combo pack with polyethylene P8-6, or equivalent.

(m) *Culture tubes.*—10 mL borosilicate, 13 × 10 mm, Corning No. 99445, or equivalent.

(n) *Pasteur pipettes.*—Kimble No. 72050, or equivalent.

(o) *Columns, SPE octadecyl C_{18}.*—3 mL capacity, 40 μm average particle diameter, 60 Å pore size, 17% C loading, Baker No. 7020-3, or equivalent.

(p) *Centrifuge tubes, borosilicate glass, 15 mL.*—Kimble No. 73785-15 and phenolic cap (PTFE faced rubber line), Kimble No. 73802-15415, or equivalent.

(q) *Gas chromatograph.*—Hewlett-Packard 5880A, or equivalent, equipped with a capillary inlet (splitless injection), an EC 63Ni detector. The column was a 30 m DB-1 column with a 0.254 mm id and a film thickness of 0.25 μm (J&W Scientific, or equivalent).

(r) *Gas chromatograph/mass spectrometer.*—Extrel Model 275, or equivalent; quadrupole equipped with a capillary inlet (splitless injection) fitted with an OV-1, 25 m × 0.20 mm id, fused-silica cross-linked methyl silicone column with a film thickness of 0.33 μm (Hewlett-Packard 19099A-102, or equivalent), and capable of NICI.

Reagents

(a) *Ethyl acetate.*—Omni Solv EXO241-1, or equivalent.

(b) *Methanol.*—Burdick & Jackson, No. 230-4 high purity, or equivalent.

(c) *Chloroform.*—Omni Solv CX1054-1, or equivalent.

(d) *Sodium chloride.*—ACS Grade.

(e) *Hexane.*—UV grade (Burdick & Jackson, No. 216, or equivalent).

(f) *Acetonitrile.*—UV grade (Burdick & Jackson, No. 015, or equivalent).

(g) *Hexamethyldisilazane–chlorotrimethylsilane–pyridine (3 + 9 + 1).*—Supelco Sylon HTP No. 3-3-43, or equivalent.

(h) *β-Glucuronidase, type IX-A.*—Sigma G7396. Dilute with buffer (i) to 4000 units/mL. Prepare fresh daily.

(i) *Phosphate buffer solution.*—0.1M KH_2PO_4 and Na_2PO_4 (ACS reagent grade) aqueous, pH 6.8 ± 0.1, adjusted to 6.8 with appropriate reagent.

(j) *Cyclohexane, pesticide grade.*—Fisher C553, or equivalent.

(k) *Water, high purity.*—18 MΩ resistance.

(l) *CAP.*—Sigma Chemical, 99% pure.

(m) *m-CAP.*—USDA, FSIS Midwestern Lab., PO Box 5080, St. Louis, MO 63115. Synthesized for FSIS by Richard K. Hill at the University of Georgia (13).

(n) *Perfluorotributylamine.*—Sigma Chemical.

Preparation of Standards

(a) *CAP.*—*(1) Stock A (500 μg/mL).*—Weigh 50 mg of CAP in a 100 mL volumetric flask. Dissolve standard and dilute with methanol. *(2) Intermediate solution (50 μg/mL).*—Dilute 10 mL of stock A to 100 mL in a volumetric flask. *(3) Working solution (100 ng/mL).*—Transfer 200 μL of the intermediate solution to a 100 mL flask and dilute to volume with methanol. Store all solutions in amber flasks at 4°C. Stock and intermediate solutions are prepared fresh every 6 months, and working solutions are prepared monthly.

(b) *m-CAP.*—*(1) Stock B (500 μg/mL).*—Weigh 50 mg of *m*-CAP in a 100 mL volumetric flask. Dissolve standard and dilute with methanol. *(2) Intermediate solution (50 μg/mL).*—Dilute 10 mL of stock B to 100 mL in a volumetric flask. *(3) Work solution (100 ng/mL).*—Transfer 200 μL of the intermediate solution to a 100 mL flask and dilute to volume with methanol. Store all solutions in amber flasks at 4°C. Stock and intermediate solutions are prepared fresh every 6 months, and working solutions are prepared monthly.

Extraction and Cleanup

Weigh 10 g of ground calf muscle tissue into a 50 mL centrifuge tube. To each sample, add 100 μL of *m*-CAP (100 ng/mL; 1 ppb).

To prepare fortification curve, prepare 1 blank and 3 fortified calf muscle samples to be analyzed with each sample set. Add the *m*-CAP at 1 ppb equivalence (100 ng/mL) to each sample. Fortify 1 sample each at 0.5 ppb (50 μL working standard), 1.0 ppb (100 μL working standard), and 2.0 ppb (200 μL working standard). Data generated from the fortified standards will be used for calculations.

Add 15 mL of pH 6.8 phosphate buffer and 200 μL of β-glucuronidase (800 units) to all blanks, fortification samples, and samples for analysis. Blend with a Tissuemizer for 30–60 s at room temperature. Incubate all tubes for 90 min at 37°C. After incubation, the samples may be left in the refrigerator overnight. Equilibrate samples to room temperature. Add 15 mL of ethyl acetate to each tube. Mix the tubes on a vortex mixer for 30 s to extract *m*-CAP and CAP. Centrifuge at 2000 rpm (1000 × *g*) for 2 min to separate the phases. Remove the ethyl acetate (upper) phase with a disposable pipette and transfer to a clean 50 mL tube. Repeat the ethyl acetate extraction steps a second time. Reduce the ethyl acetate volume to 1 mL on an N-Evap under a gentle stream of nitrogen, using a sand bath at ca 60°C. Add 4 mL of aqueous NaCl to all tubes and mix on vortex mixer for 5–10 s. Continue evaporation of ethyl acetate on an N-Evap until the ethyl acetate layer is absent and an oily residue is left. Add 5 mL of hexane to the 4 mL of aqueous 4% NaCl layer. Mix on a vortex mixer for 10 s and centrifuge at 2000 rpm (1000 × *g*) for 1 min. Remove the top layer and discard, and then repeat the hexane partition.

Prepare a C_{18} cartridge for each sample, blank, and fortified control by washing the C_{18} column sequentially with 5 mL of methanol, 5 mL of chloroform, 5 mL of methanol, and 10 mL of distilled water. Discard all washes. Load the entire aqueous extract onto a C_{18} column with a disposable Pasteur pipette. Discard the eluate. Rinse the sample by mixing on a vortex mixer twice with 1 mL of distilled water and adding the rinses onto the C_{18} column with 1 mL of water followed by 2 mL of methanol–water (20 + 80). Let the last wash elute completely through the column and discard the washes.

Elute the CAP from the C_{18} column with acetonitrile (2 × 1.5 mL) and collect the eluate in a clean 10 mL culture tube. Evaporate the acetonitrile eluate to ca 0.5 mL (not to dryness!) on a sand bath at 60°C and a gentle stream of nitrogen. Transfer the extract to a 1 mL conical vial. Rinse the 10 mL tube by mixing on a vortex mixer for 5 s with 0.5 mL of acetonitrile and add the washing to the 1 mL vial. Evaporate to dryness gently with nitrogen on a Reacti-Therm at 60°C.

Caution: Avoid moisture from this point forward.

To the dried residue, add 200 µL of Sylon HTP, stopper, and mix on a vortex mixer for 5 s. Reduce at 60–70°C with a gentle stream of nitrogen to ca 10 µL. *Caution: Excessive dryness may result in loss of analyte.* Reconstitute the residue in 100 µL of cyclohexane–hexane (60 + 40) and mix on a vortex mixer for 5 s. Inject 2–5 µL of derivatized dimethylsilyl material into GC system for quantitative determination or a GC/MS system for confirmation.

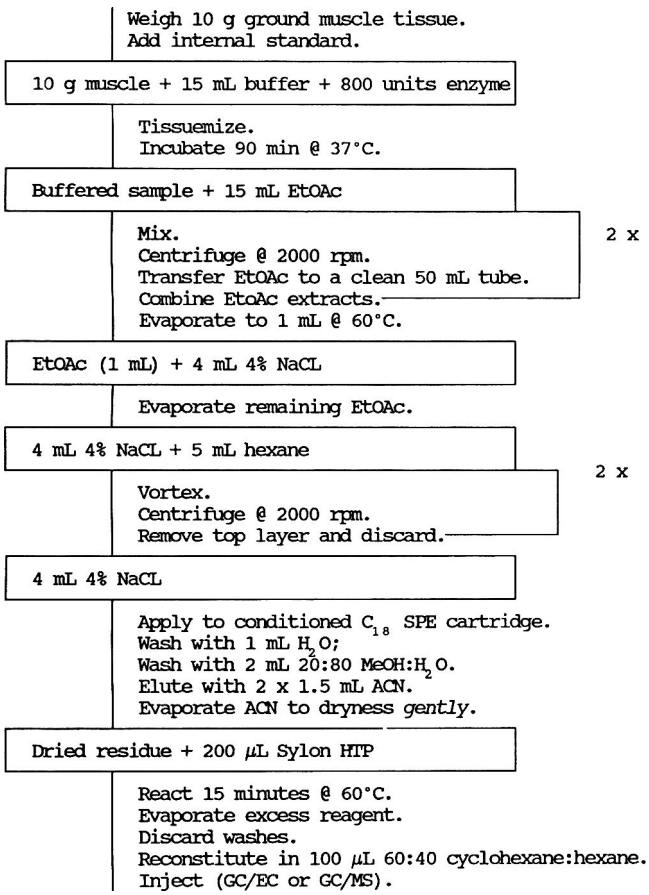


Figure 2. Flow chart of extraction procedure.

Table 1. Gas chromatographic conditions[a] for the determination of chloramphenicol

Parameter	Setting
Carrier gas	Helium; linear velocity, 29 cm/s
Makeup gas	Argon–methane (95 + 5); flow rate, 50 mL/min
Initial column temp.	80°C, hold for 1 min
Temperature program	Programmed at 30°C/min to 260°C; hold for 10 min or until *m*-CAP and CAP have eluted; programmed at 30°C/min to 300°C; hold for 5 min to make sure all the sample has eluted
Injector temperature	280°C
Detector temperature	350°C
Sensitivity setting	2^8 attenuation
Expected response	50% full-scale deflection for 0.20 ng CAP

[a] These conditions are for a Hewlett-Packard 5880A GC described in *Apparatus* (**q**) and are given as an example only. The analyst should optimize these parameters for the instrument being used.

Figure 2 is a flow chart summary of the extraction. The GC operating conditions for chromatography are listed in Table 1.

Calculations

m-CAP is used as an internal standard for calculating CAP concentration. By an acceptable means, measure the peak height or area for both CAP and *m*-CAP in the samples fortified at 0.5, 1.0, and 2.0 ppb that have been processed through the extraction procedure. Calculate the respective ratios for CAP by dividing the area or height by the corresponding *m*-CAP area or height. Using the ratios and associated part-per-billion values, calculate a linear-regression calibration curve by least-squares computation. The calibration curve is acceptable if the correlation coefficient, *r*, is greater than 0.9945.

Representative chromatograms from analysis of blank tissue and fortified muscle tissue are shown in Figure 3.

Confirmation

The chromatographic and instrumental parameters for confirming the presence of CAP are listed in Table 2. The following criteria are used to determine confirmation. The retention times of a known CAP standard fortified in tissue and the sample in question should be within 5%. (Although a 5% variation in retention is generous for a capillary column, the wide retention time window was necessary because the glass interface for the mass spectrometer is not insulated and retention times are difficult to reproduce.) As an example using the conditions listed in Table 2, CAP elutes in 9.0 min; therefore, ± 0.5 min is acceptable.

The 4 ions monitored are *m/z* 468, 466, 322, and 304. For confirmation, compute 3 ratios for a known CAP standard, fortified muscle sample, and the sample in question as follows: 468/466, 322/466, and 304/466. For successful confirmation of

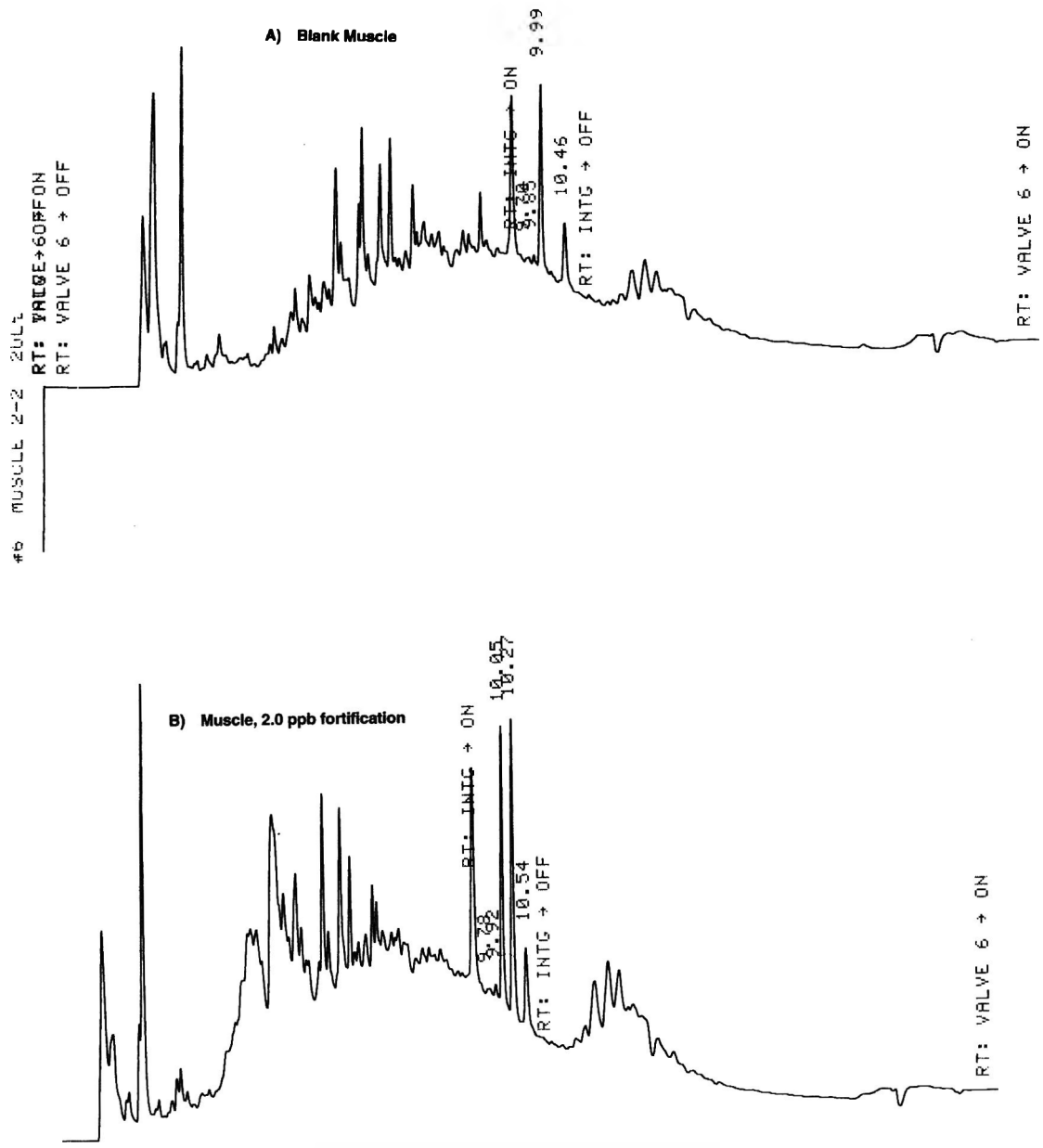


Figure 3. (a) Chromatogram of blank bovine muscle in cyclohexane–hexane (60 + 40). No response is noted for CAP at ca 10.21 min.; for *m*-CAP at 1 ppb, the retention time is ca 9.99 min. (b) Chromatogram of fortified CAP at 10.27 at 1 ppb in bovine muscle in cyclohexane–hexane (60 + 40). *M*-CAP retention time is 10.05 min.

a presumptive positive quantitated between 0.50 and 1.0 ppb, the 4 ions must be present with retention times equivalent to that found for a known CAP standard fortified in tissue. In addition, the 466/468 ratio and 1 other ion ratio should be within ±20% of the fortified muscle tissue.

A high-source temperature (approximately 260°C) is necessary to obtain the *m/z* 322 and 304 ions. At lower source temperatures (approximately 140–170°C), the molecular ion cluster and the M-$(CH_3)_3SiOH$ ion cluster have been used for confirmation in some methods (14, 15). The higher source temperature was used to produce more ion fragments for confirmation. At higher CAP concentrations, the *m/z* 358 ion has also been monitored (7). NICIMS confirmation monitoring the *m/z* 468, 466, 322, and 304 ions for confirmation of CAP in milk is also being developed in The Netherlands (16).

Discussion

CAP analytical data for calf muscle are listed in Table 3. Consensus means (X), coefficients of variation for repeatability (CV_o), reproducibility (CV_x), and bias (CV_b) for all laboratories are listed. Laboratory 2 exhibited the lowest CV_o (1.0–

Table 2. Instrumentation parameters[a] for confirmation procedure

Instrument	Parameter	Condition or Setting
Gas chromatograph	Column	OV-1 fused silica capillary, 25 meter
	Injector	230°C
	Temperature programming	150°C with no initial hold, programmed to 300°C at 20°C/min, final hold for 10 min
	Transfer line temp.	300°C
	Helium flow rate	29 cm/s, splitless injection
Mass spectrometer	Detection	Enhanced negative ion chemical ionization
	Ionization gas	Isobutane or methane
	Source pressure	1.0×10^{-5}T
	Source temperature	260°C
	Mode of Operation	NICI-SIM
	Dwell time	10 ms
	Calibration standard	PFTBA; maximize on *m/z* 452
	Scan width	0.2 amu
	Expected signal/noise ratio of lowest intensity ion	10/1
	Filament voltage	300 eV
	Filament current	1000 μA

[a] The following conditions are for the Extrel instrument described in *Apparatus* (r) and are given as an example only. The analyst should optimize the instrumental parameters for the instrument being used.

Table 3. Validation study in bovine muscle, quantitative determinations (ppb)[a]

		Level fortified, ppb				Incurred, ppb		
Lab.	Day	0	0.6	1.5	2.4	US_{Lo}	US_{Hi}	A
1	1	0.0	0.61	1.61	2.50	1.01	2.67	0.114
	2	0.08	1.05	0.84	1.64	1.20	1.68	0.242
	3	0.0	0.44	1.98	1.88	1.42	2.93	0.152
	X	NC	0.70	1.48	2.01	1.21	2.43	0.17
	CV_o	NC	45.0	39.4	22.1	17.0	27.2	38.8
2	1	0.0	0.57	1.49	2.27	1.86	2.91	0.48
	2	0.0	0.59	1.61	2.31	1.77	3.07	0.58
	3	0.0	0.54	1.34	2.31	1.62	2.94	0.51
	X	NC	0.57	1.48	2.23	1.75	2.97	0.52
	CV_o	NC	4.4	9.1	1.0	6.9	2.9	9.8
3	1	0.0	0.47	1.15	2.07	1.48	1.99	0.42
	2	0.0	0.38	1.45	1.58	1.43	1.70	0.43
	3	0.0	0.57	1.12	1.92	1.71	2.09	0.28
	X	NC	0.47	1.24	1.86	1.54	1.93	0.38
	CV_o	NC	20.1	14.7	13.5	9.7	10.5	22.3
4	1	NV	0.59	1.50	1.92	1.76	2.43	0.55
	2	0.16	0.65	1.95	2.71	1.57	2.06	0.57
	3	0.0	NV	1.41	2.25	1.49	1.78	0.51
	X	NC	0.62	1.62	2.29	1.61	2.09	0.54
	CV_o	NC	6.8	17.6	17.3	8.6	15.6	5.6
5	1	0.0	0.53	1.22	NV	1.42	3.84	0.38
	2	0.0	0.85	1.55	2.64	1.44	2.41	NV
	3	0.0	0.64	1.49	2.67	1.55	3.21	0.55
	X	NC	0.67	1.42	2.66	1.49	3.15	0.47
	CV_o	NC	24.2	12.4	0.8	4.8	22.7	25.9
All	X	NC	0.61	1.45	2.19	1.52	2.51	0.41
	CV_o	NC	28.7	22.0	13.9	9.5	18.7	17.0
	CV_x	NC	27.9	20.5	16.8	14.6	25.3	38.0
	CV_b	NC	22.4	16.2	14.8	13.5	22.9	36.7

[a] X, mean value; CV_o, repeatability coefficient of variation; CV_x, reproducibility coefficient of variation; CV_b, coefficient of variation for laboratory bias; NV, no value reported; NC, not calculated; US, U.S. incurred bovine muscle (Hi, high dose; Lo, low dose); A, Australian incurred bovine muscle.

9.8%), whereas Laboratory 1 generally showed the highest CV_o (17–45%). For the other 3 laboratories, CV_o values ranged from 0.8 to 25.9%. For all 5 laboratories, overall CV_o values ranged from 13.9 to 28.7% for tissues fortified in the 0.6–2.4 ppb range. For the 3 incurred tissues, the CV_o values varied between 9.5 and 18.7% over a CAP concentration range of 0.48 to 2.5 ppb.

For the fortified tissues, the overall repeatability decreased with concentration from 28.7 to 13.9% over the 0.61 to 2.19 ppb range. The CV_x values decreased from 27.9 to 16.8% over the CAP fortified tissue range of 0.61 to 2.19 ppb. For the 3 incurred tissues, the CV_x values varied from 14.6 to 38% over the 0.48 to 2.5 ppb range. The CV_x values for both the fortified and incurred CAP muscles tissues were equivalent, a result clearly supporting the performance of the method. Except for the Australian incurred sample, the CV_x values and the CV_b values are similar. This finding confirms that generally each laboratory can successfully duplicate a test.

Laboratories 1 and 4 each reported 1 low CAP value for a blank. However, 1 false positive, the 0.08 ppb value from Laboratory 1 was not confirmed by mass spectrometry. Because Laboratory 4 did not participate in the confirmation part of the study, it cannot be determined whether the presumptive finding is a false positive. Table 4 lists the confirmation results from the 3 participating laboratories. There were no positive confirmations on the 4 blank CAP tissues subjected to confirmation in 2 laboratories. All 6 of the 0.6 ppb samples subjected to confirmation were confirmed on the basis of the criteria outlined. Laboratory 1 could not confirm CAP in the 0.48 ppb incurred Australian muscle tissue. However, Laboratories 2 and 5 suc-

Table 4. Chloramphenicol validation study in bovine muscle, GC/NICIMS confirmation[a]

		Theoretical level fortified, ppb[b]		Incurred, ppb		
Lab.	Day	0	0.60	1.52[c]	2.51[d]	0.48[e]
1	1	–	C	C	C	NC
	2	NC	–	C	C	–
	3	NC	C	C	–	NC
2	1	–	C	C	C	C
	2	NC	–	C	C	–
	3	NC	C	C	–	C
5	1	–	C	C	NA	C
	2	NA	–	NA	C	–
	3	NA	C	NA	–	C

[a] NC, not confirmed; C, confirmed; NA, not attempted; –, no confirmation requested.
[b] Mean values = 0 and 0.61 ppb, respectively.
[c] U.S. incurred bovine muscle, low dose.
[d] U.S. incurred bovine muscle, high dose.
[e] Australian incurred bovine muscle.

cessfully confirmed the designated Australian incurred samples.

The results of the 5-laboratory study indicate that the GC quantitative procedure for the determination of CAP in bovine muscle is acceptable as an assay from 0.5 to 2.5 ppb CAP. Also, the confirmation procedure using NICIMS successfully confirmed samples ≥0.6 ppb CAP with no false positives in the blank tissue matrix.

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DRUGS, COSMETICS, FORENSIC SCIENCES

Determination of Semduramicin Sodium in Poultry Liver by Liquid Chromatography with Vanillin Postcolumn Derivatization

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A liquid chromatographic (LC) method is described for the quantitative determination of semduramicin sodium in broiler liver when administered under projected use conditions. For this procedure, semduramicin sodium is extracted from liver with methanolic ammonium hydroxide, separated and concentrated by solid-phase extraction steps, and determined by LC with postcolumn derivatization with vanillin. The mean recovery of drug was 95% over the 40–320 ng/g range, the coefficient of variation was ±10% or better, and no interference was observed from commercial polyether ionophores. The minimum level of detection for semduramicin sodium in broiler liver is 25 ng/g.

Semduramicin (Figure 1) is a new potent monocarboxylic polyether antibiotic with anticoccidial activity that is to be administered in feeds in the 15–30 ppm range (1, 2). Its structure and ionophoric properties are comparable with several widely used polyether antibiotics (3, 4), including the commercialized anticoccidial feed additives monensin, salinomycin, lasalocid, narasin, and maduramicin. The discovery of semduramicin arose from an effort to identify fermentation-derived products for the treatment of coccidiosis in poultry (5).

Previous studies defined the metabolism and depletion of residues of semduramicin sodium in poultry when given under projected use conditions (6). After administration of [^{14}C]-semduramicin sodium in feed at 25 ppm, the tissue containing the highest total residues at all withdrawal times was the liver. Mean total residues in liver decreased from 273 ng/g at 6 h of withdrawal to 58 ng/g at 24 h (6). Analysis and profiling of residues in liver also revealed that unchanged semduramicin sodium was the major component of total radioactivity. It is for this reason that the unchanged drug was selected as the marker (7, 8) for monitoring depletion of residues of semduramicin sodium.

Several reports have covered the development of liquid chromatographic (LC) methods with postcolumn derivatization with vanillin for the determination of polyether ionophores in premixes and feeds (9–12). We adopted and extended this derivatization reaction to the determination of semduramicin sodium in poultry tissues. The low detection limit and enhanced selectivity of the method are illustrated in this report.

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Experimental

Principle

Semduramicin sodium is extracted from liver with 1% NH_4OH in methanol–water, separated from coextractives by reversed- (C_8) and normal- (silica gel) phase extraction columns, and determined by LC with postcolumn derivatization. The ionophore is chromatographed on a normal-phase column and is baseline resolved from tissue coextractives within a total run time of ca 24 min.

To quantify semduramicin in purified poultry liver extracts, the LC procedure relies on a postcolumn reactor with vanillin (4-hydroxy-3-methoxybenzaldehyde) as a derivatizing reagent (13). The chromophore is formed under anhydrous, acidic conditions in which the vanillin reacts with the hydroxyl groups of semduramicin to yield a derivative that strongly absorbs at 522 nm. Heat is required to drive the reaction, but the required temperature is easily achieved in the LC system.

For quantitation, peak heights of semduramicin sodium derived from calibration and fortified samples are measured on a recording integrator to define a standard curve for recovery of the drug. Concentrations of unknowns are determined by reference to a calibration curve with a correction for the recovery of semduramicin sodium.

Results of semduramicin concentrations in incurred liver tissues obtained from radiotracer studies using this procedure were confirmed by a reverse isotope dilution assay and by metabolic profiling techniques (6).

Reagents

(a) *Solvents and chemicals.*—Ethyl acetate, ACS grade, Aldrich PN 31,990-2 (use no substitutes); isooctane, high purity, Burdick & Jackson PN 362; triethylamine, 99.9%, Kodak PN 616; glacial acetic acid, ACS, Fisher Scientific PN A-38 S; sulfuric acid, ACS, Fisher Scientific PN A-300 S; methanol, LC grade, Fisher Scientific PN A452-4; vanillin, 99%, Mallinckrodt PN 2759 (use no substitutes); ethyl alcohol, 200 proof, USI Chemicals Co.; chloroform, ACS, Fisher Scientific PN C298-4; methylene chloride, ACS, Fisher Scientific PN D37-4; ammonium hydroxide, GR EM Science, PN AX1303P-1; distilled LC water, Burdick & Jackson PN 365-4.

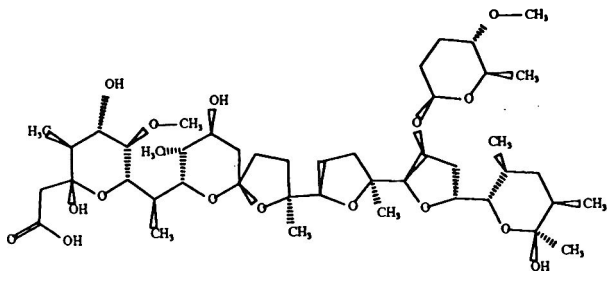

Figure 1. Structure of semduramicin.

(**b**) *LC mobile phase (filter and degas).*—Ethyl acetate–isooctane–glacial acetic acid–triethylamine–methanol (650 + 350 + 4 + 2 + 1); prepare by adding the specified amounts to a liter flask, filter, and degas by vacuum.

(**c**) *Vanillin reagent for postcolumn derivatization.*—Protect from light; filter, degas, and prepare every other day. (*1*) Add 20 mL of sulfuric acid to 500 mL of ethyl alcohol; mix well and place at –20°C for 15 min to bring back to room temperature. (*2*) Add 30 g of vanillin to 500 mL of ethyl alcohol. Mix each component (*1*) and (*2*) and then mix together, filter and degas, and protect from light by wrapping flask with aluminum foil.

(**d**) *Solutions.*—Extraction solution: 1% NH_4OH in methanol–water (8 + 2), prepare by adding 5 mL of NH_4OH to 500 mL of 8:2 methanol–LC distilled water; C_8 BondElut wash: methanol–LC distilled water (25 + 75); reconstitution solvent for silica BondElut methylene chloride–isooctane (1 + 1); silica BondElut elution solvent: methylene chloride–methanol (18 + 2); reconstitution solvent for LC injection: ethyl acetate–isooctane (4 + 6).

Apparatus

(**a**) *LC equipment.*—Mobile-phase pump, Waters Assoc. model 6000A; reagent pump, Milton Roy Model ConstaMetric III; pulse dampers for reagent pump: high pressure (Waters Associates, PN 25552), low pressure (Waters, PN 98060), restrictor, solvent delivery system (Waters, PN 25561), analytical column (Polymer Labs PN PLRP-S 5 μ 100 A); in-line solvent filter for reagent pump (Scientific Systems, Inc.) PN 05-0105; analytical LC column, DuPont Zorbax silica, 4.6 mm × 25 cm, PN 880952-701; guard column, Supelco Inc., LC-Si 2 cm, 40 μ, PN 5-8951; mixing tee, S.S.I. PN 01-0165; reaction coil, stainless steel tubing 0.01 in. id × 50 ft; stir plate, Thermolyne type 1000; silicone oil bath; temperature controller, Ace Glass cat. No. 12106-10; signal filter, Spectrum Model 921; UV detector, Kratos Analytical Model Spectroflow 757 with tungsten lamp; integrator, Spectraphysics Model 4400; autoinjector, Perkin Elmer Model ISS-100; adjustable back pressure regulator, Rainen PN 02-0176.

(**b**) *Sample preparation equipment.*—Food processor, Black & Decker Handy Chopper PN HC-20; multitube vortex mixer, Scientific Products Cat. No. S8215-1; BondElut manifold, American Bioanalytical VacElut SPS24; BondElut LRC C_8 200 mg, Varian PN 1211-3025; BondElut LRC silica 500 mg, Varian PN 1211-3036; borosilicate disposable culture tube, 16 × 125 mm; borosilicate disposable culture tube, 16 × 100 mm; disposable centrifuge tubes, 15 mL, 17 × 125 mm.

LC Conditions

Equilibrate the LC system for at least 2 h prior to running samples. More time may be required during the initial setup of the LC–postcolumn derivatization system. The flow rates for the mobile phase and vanillin are 0.6 and 0.3 mL/min, respectively. Maintain the oil bath at 95 ± 1°C. Set the detector at 522 nm with 0.005 AUFS. Filter cut-off frequency is 0.01 Hz. Set the integrator with an attenuation of 8, peak width of 12, peak threshold of 1500, and chart speed of 0.5 cm/min. Use peak height integration. Total run time is approximately 24 min. At the end of each sample run prior to shutdown, flush the system with methanol flowing through the vanillin pump and ethyl acetate–isooctane (6 + 4) flowing through the mobile-phase pump. Rinse the system for at least 3 h. Maintain care in activating the mobile-phase pump prior to the reagent pump to prevent the vanillin reagent (methanol rinse) from entering the analytical column. During system shutdown, shut off the pumps in reverse order: vanillin pump first followed by the mobile-phase pump.

Procedure

(**a**) *Extraction of semduramicin from liver.*—Homogenize liver samples in a food processor to ensure a homogeneous sample. The sample homogenate should be smooth and void of any chunks. Tare a 16 × 125 mm disposable culture tube on a top-loading balance. Weigh the sample by dispensing approximately 1.25 g of the homogenate into the lower half of the disposable tube by using a 3 mL B&D disposable syringe. Add 7.5 mL of 1% NH_4OH in methanol–water (8 + 2), mix (vortex mixer) for 3.0 min, and incubate at 55°C of 1 h. Centrifuge the sample for 5 min at 4000 rpm and decant the supernatant into a clean disposable culture tube; rinse the first tube with 1–2 mL of methanol, being careful not to disturb the sediment, and combine with the supernatant. Reduce the volume to ca 2–3 mL under N_2 at 55°C; the supernatant will become clear yellow. Add 5.0 mL of distilled water. (At this point, samples may be stored overnight in a capped tube in a refrigerator for further processing on the following day.) Mix the aqueous samples (vortex mixer) and sonicate for 5 min prior to applying to solid-phase extraction columns.

(**b**) *Solid-phase extraction of tissue extract.*—Prepare the C_8 BondElut by washing with 5 mL of acetonitrile followed by 5 mL of methanol and then 5 mL of distilled water; apply 100 μL of distilled water to the columns without vacuum to prevent the column from drying out. Mix the sample (vortex mixer) and apply to the extraction column by directly pouring into the C_8 reservoir. Rinse the sample tube with 1 to 2 mL of water and apply the rinse as well. Process the sample through the C_8 BondElut by washing the cartridge with 3 mL of distilled water followed by 1.0 mL of methanol–water (25 + 75). Elute the sample with 5 mL of ethyl acetate and evaporate to dryness under N_2 at 55°C. Reconstitute the residue with 6 mL of methylene chloride–isooctane (1 + 1), mix (vortex mixer),

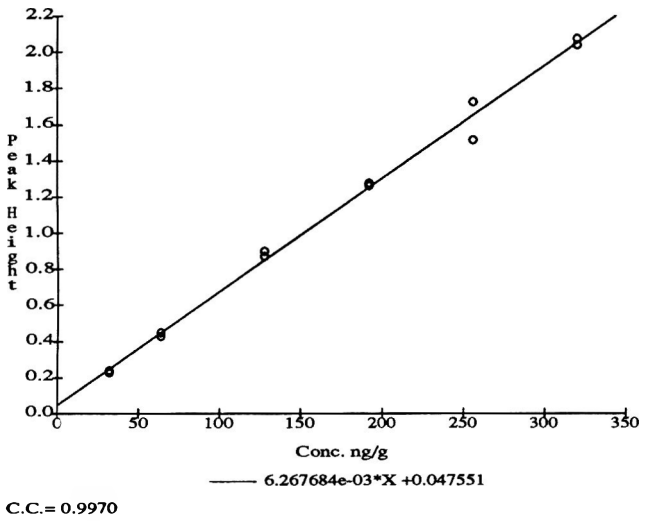

Figure 2. Semduramicin calibration curve, 32–320 ng/g.

and sonicate for 5 min; apply this solution to a silica BondElut. The silica BondElut is prepared with 5 mL of chloroform followed by 5 mL of methylene chloride–isooctane (1 + 1). Approximately 0.1 mL of methylene chloride–isooctane (1 + 1) is added without vacuum to the top of the packing to prevent the column from drying out. Rinse the sample tube with 1.5 mL of methylene chloride–isooctane (1 + 1) and transfer to the silica BondElut. Process the sample through the silica BondElut by washing the cartridge with 2.5 mL of methylene chloride–isooctane (1 + 1) followed by 1.0 mL of ethyl acetate. Elute the sample into a disposable centrifuge tube with 5 mL of methylene chloride–methanol (18 + 2). Evaporate the eluant to dryness under N_2 at 55°C.

(c) *LC of tissue extract.*—Reconstitute the dried residue with 150 µL of ethyl acetate–isooctane (4 + 6). Cap, mix (vortex mixer), and sonicate the tubes for 2 min prior to injection. Inject 75 µL onto the LC.

(d) *Calibration curve (equivalent to 16, 32, 64, 128, 192, 256 and 320 ng/g in tissue).*—Construct a calibration curve by injecting 5, 10, 20, 40, 60, 80, and 100 µL of a 2.0 µg/mL calibration standard or 10, 20, 40, 80, 120, 160, and 200 ng of semduramicin sodium. Standardization is based on the analysis of a 1.25 g sample, reconstitution of the final residue with 150 µL of solvent, and injection of 75 µL. Inject calibration standard in duplicate. Construct the calibration curve by relating peak height measurements to the equivalent concentration in tissue and using the slope and intercept derived by linear-regression analysis. The calibration curve must contain at least 5 points selected on the basis of expected tissue concentrations.

Precautions

A BondElut vacuum manifold is used at all BondElut steps. Large-reservoir BondEluts are used so that the entire wash or sample can be applied in one step. BondEluts with adapters and glass syringes should be used in place of the large-reservoir BondEluts because drug recovery may be compromised.

Control liver homogenate should be assayed prior to running unknown samples to ascertain the presence of late-eluting

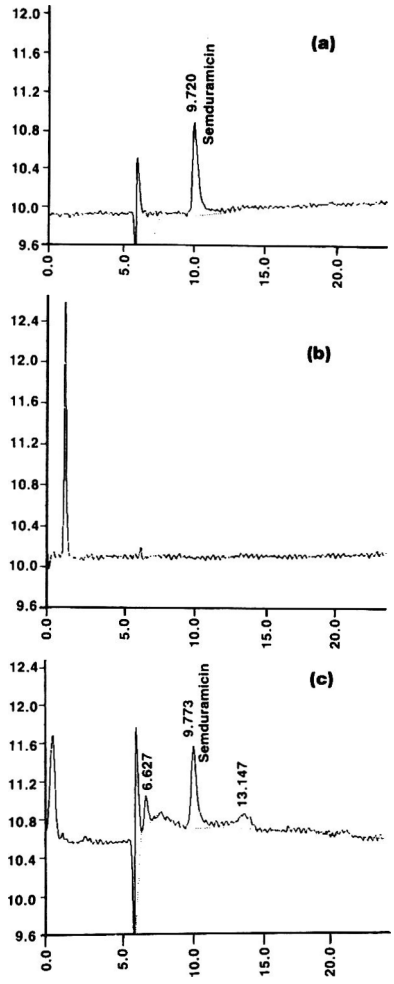

Figure 3. Chromatograms of (a) 128 ng of a calibratio standard of semduramicin sodium, (b) control chicken liver extract, and (c) an extract of chicken liver fortified with 120 ng/g of semduramicin sodium. See text for LC conditions. Semduramicin retention time is ca 11.3 min.

endogenous peaks at 35 and 53 min. Although late-eluting peaks may be minor, retention times may vary and may interfere with the drug peak if an appropriate run time is not selected. In most instances, a 24 min run time is sufficient.

Except for ethyl acetate and vanillin, specified solvents, chemicals, supplies, and equipment can be substituted with equivalent items. However, a poor quality of ethyl acetate will result in chromatographic anomalies such as increased retention time and adsorption of drug to the column. A poor quality of vanillin will result in decreased vanillin response and shorter stability of the prepared vanillin reagent.

Each set of fortified and withdrawal samples should be processed with the same lots of BondElut cartridges.

The use of ethyl acetate–isooctane (4 + 6) is recommended for preparing the calibration standard and reconstituting residues to ensure linearity of the standard curve.

Minor leaks at fittings may cause substantial baseline noise. All leaks should be repaired prior to assaying samples.

Reaction coil temperatures greater than 100°C may cause the mobile phase to boil and result in baseline spiking and increased noise.

The low-pass electronic noise filter is required for optimum sensitivity and low baseline noise. If low sensitivities are not required (>100 ng/g), a noise filter may not be necessary if the 50 ft × 0.01 in. id coil is substituted with a 25 ft × 0.02 in. id coil.

Calculations

Determine sample and fortified liver concentrations (ng/g) by using the slope and intercept values from the calibration curve. Adjust sample concentration for the amount of sample initially weighed and for the percent recovery.

$$\text{Sample conc. (ng/g)} = \frac{\text{sample peak height} - \text{intercept}}{\text{slope}} \times \frac{\text{target weight}}{\text{amount weighed}} \times \frac{1}{\text{mean recovery}}$$

where

$$\text{Recovery} = \frac{\text{fortified standard peak height} - \text{intercept}}{\text{slope}} \times \frac{1}{\text{target concentration}}$$

Table 1. Semduramicin sodium found (ng/g) in fortified poultry liver samples

	Semduramicin sodium found (ng/g) in sample fortified at indicated level										
Control Liver	25	40	50	80	100	120	150	200	240	300	320
0	27	36	50	81	97	124	145	229	249	292	308
0	—	39	51	78	93	129	146	194	244	301	288
0	—	40	49	79	95	121	149	196	240	288	319
0	—	40	50	73	113	119	141	201	209	312	325
0	—	40	—	77	92	134	—	—	223	—	—
0	—	44	—	76	92	117	—	—	217	—	—
0	—	46	—	83	94	118	—	—	224	—	—
—	—	46	—	82	99	118	—	—	225	—	—
—	—	47	—	82	89	117	—	—	—	—	—
—	—	—	—	81	85	114	—	—	—	—	—
—	—	—	—	—	86	—	—	—	—	—	—
—	—	—	—	—	74	—	—	—	—	—	—
—	—	—	—	—	105	—	—	—	—	—	—
—	—	—	—	—	106	—	—	—	—	—	—
—	—	—	—	—	105	—	—	—	—	—	—
—	—	—	—	—	111	—	—	—	—	—	—
—	—	—	—	—	108	—	—	—	—	—	—
—	—	—	—	—	120	—	—	—	—	—	—
—	—	—	—	—	114	—	—	—	—	—	—
—	—	—	—	—	116	—	—	—	—	—	—
—	—	—	—	—	103	—	—	—	—	—	—
—	—	—	—	—	105	—	—	—	—	—	—
—	—	—	—	—	96	—	—	—	—	—	—
—	—	—	—	—	95	—	—	—	—	—	—
—	—	—	—	—	101	—	—	—	—	—	—
—	—	—	—	—	102	—	—	—	—	—	—
—	—	—	—	—	107	—	—	—	—	—	—
—	—	—	—	—	100	—	—	—	—	—	—
—	—	—	—	—	98	—	—	—	—	—	—
—	—	—	—	—	102	—	—	—	—	—	—
Mean	27	42	50	79	100	121	145	205	229	298	310
Standard deviation	—	3.8	1.0	3.3	10.0	6.1	3.2	16.3	14.0	10.7	16.3
Coefficient of variation	—	8.9	2.1	4.2	9.9	5.1	2.2	7.9	6.1	3.6	5.2

Table 2. Recovery data for semduramicin sodium broiler liver assay

Amount added, ng/g	Amount found, ng/g	No. of replicates	Standard deviation	Coefficient of variation	Recovery, %
25	25	1	—	—	100
40	39	9	2.7	6.9	98
50	48	4	1.3	2.7	96
80	73	10	2.7	3.7	91
100	98	30	10.1	10.3	98
120	111	10	7.7	6.9	93
150	139	4	6.7	4.8	93
200	196	4	13.3	6.8	98
240	210	8	17.4	8.3	88
300	285	4	17.3	6.1	95
320	292	4	15.4	5.3	91
				Mean	95
				Standard deviation	3.7

Results

The method described in this report is a modification of an earlier procedure used to assay tissue over the 10–300 ng/g range with a mean recovery of 84.3% (range, 70.9–118.4%). Changes to the earlier procedure included the incorporation of precautionary statements into the procedure to reflect experience with a collaborator and improvement of the overall recovery of semduramicin sodium by modification of the tissue extraction step and lowering of the liver sample size from 2.5 to 1.25 g. The assay is linear from 32 to 320 ppb and has a correlation coefficient of >0.99 (Figure 2). Chicken liver fortified with semduramicin sodium over the range of 25 to 320 ng/g and assayed in replicate gave results close to nominal levels at all concentrations, with a relative standard deviation of $\leq \pm$ 10% (Table 1). The recovery of semduramicin sodium ranged from 88 to 98%, with a mean of 95% and a coefficient of variation of $\pm$ 3.7% over the range of 40 to 320 ng/g (Table 2). Typical chromatograms for calibration, blank, and fortified tissue samples are given in Figure 3. The limit of detection is 25 ng/g when the signal-to-noise ratio is approximately 5/1. The sensitivity may be increased to as low as 10 ng/g with some loss of recovery by increasing sample size.

This procedure was also validated with respect of use of different lots of C_8 and silica BondEluts. In this evaluation, 6 lots of C_8 and 2 lots of silica BondElut were evaluated for the recovery of semduramicin sodium at the 100 ng/g fortification level. Mean recoveries for use of various lots ranged from 82 to 107% (Table 3), indicating that the same lots should be used when processing a set of samples.

The specificity of the assay for semduramicin sodium relative to interference by other commercial microbial ionophores was evaluated under the LC conditions described. When 100 ng of semduramicin sodium was coinjected with 50 ng of monensin, narasin, salinomycin, maduramicin, or lasalocid, no interference was observed (Figure 4). Under the conditions of the assay, maduramicin, monensin, narasin, salinomycin, and semduramicin sodium elute with retention times of 8.5, 9.7, 9.9, 10.4, and 11.4 min, respectively. Enhanced specificity for semduramicin relative to other polyether ionophores and their metabolites or degradation products may be achieved by a mass spectrometric confirmatory method (14). Five microliters of the reconstituted extract used in this LC–vanillin procedure may be applied to an atmospheric pressure ionization mass spectrometer giving additional confirmatory analysis when needed.

The stability of semduramicin sodium in frozen (–20°C) poultry liver homogenates was assessed over a 40 day storage period. In this study, 2.5 g portions of control liver homogenate were fortified with semduramicin sodium at the 60 ng/g level, and samples of each were stored at –20°C in amber glass bottles. Samples assayed in triplicate after 5, 12, 19, 26, and 40 days of storage gave respective mean levels of 56, 52, 54, 51, and 52 ng/g, with an extrapolated zero time value of 55 ng/g indicating good stability of semduramicin sodium in frozen poultry liver.

Conclusions

A sensitive and reliable LC method was developed for the quantitative determination of semduramicin sodium in broiler liver at physiologic concentrations. Consistent results were ob-

Table 3. Recovery of semduramicin sodium from poultry liver fortified at 100 ng/g with various lots of BondElut columns

C_8 BondElut lot number	Silica BondElut lot number	Mean recovery, %	Standard deviation
060892	010413-2	97	3
061063	010413-2	93	4
062091	010413-2	82	6
061713	010413-2	105	3
062423	010413-2	107	7
062753	012893-1	100	4

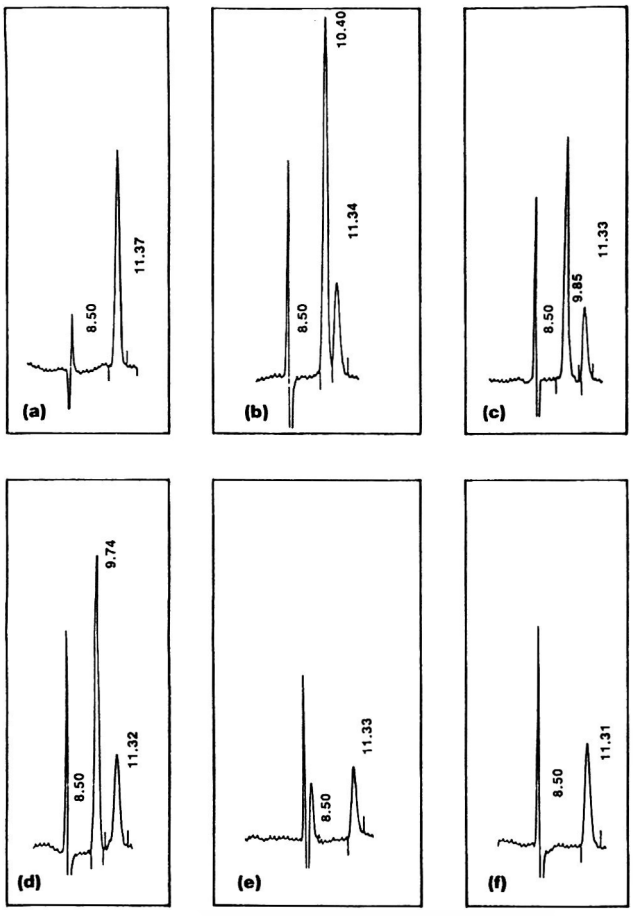

Figure 4. Chromatograms of (a) 100 ng of semduramicin sodium plus 50 ng of (b) salinomycin, (c) narasin, (d) monensin, (e) maduramicin, and (f) lasalocid. See text for LC conditions.

tained with standardized materials and calibrated equipment. Isolation of semduramicin sodium from tissue and concentration of the residue were reproducible with solid-phase extraction columns. Detection at the nanogram level and separation from coextractives in achieved by LC with vanillin postcolumn derivatization. Over the range of 40 to 320 ng/g in liver, the mean recovery of drug was 95%, and the coefficient of variation was ± 10% or better. The minimum lever of detection for semduramicin sodium in broiler liver was 25 ng/g, and under the recommended LC conditions, no interference was observed from commercial polyether ionophores.

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Gas Chromatographic Determination of *p*-Chloroaniline in a Chlorhexidine Digluconate-Containing Alcohol Foam Surgical Scrub Product

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A gas chromatographic (GC) method with flame ionization detection was developed to separate and quantitate *p*-chloroaniline (PCA) from other components in a chlorhexidine digluconate (CHG)-containing alcohol foam surgical scrub product. A simple sample preparation method was developed in which 1-butanol was used to dissolve the foam and precipitate the CHG, which otherwise would interfere with the GC analysis. The method was validated with respect to linear dynamic range, precision, accuracy, selectivity, limit of detection, and limit of quantitation.

Chlorhexidine (CH) exhibits antibacterial properties and is often used in health care skin disinfectant products such as surgical handscrubs, patient preoperative skin preparation products, and health care personnel hand-washing products (1). *p*-Chloroaniline (PCA) is a known degradation product that must be assayed in any skin care product containing chlorhexidine. Because the skin care products are usually water soluble, liquid chromatography (LC) is a favored method for PCA analysis (2–6).

If the product is not water soluble, PCA analysis by a reversed-phase LC system is not viable. A chlorhexidine digluconate (CHG)-containing alcohol foam surgical scrub product is the type of product that would not be water soluble but would require the analysis of low levels of PCA. PCA in a non-skin care product sample matrix has been analyzed by gas chromatography (GC) (7). GC analysis can be used to determine the amount of PCA present in a foam surgical scrub product, but CHG must be removed from the sample before analysis, because it can decompose in the heated injection port to produce PCA and, thus, result in inaccurate quantitation. In this work, 1-butanol was used to dissolve the sample and precipitate the CHG. A GC method was developed in which splitless injection and flame ionization detection were used to determine the amount of PCA present in a CHG-containing alcohol foam surgical scrub product.

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Experimental

Apparatus

The GC system consisted of a Varian (Walnut Creek, CA) 3400 gas chromatograph equipped with a split/splitless injector and a flame ionization detector and a Varian 8100 autosampler. The GC column was a J&W Scientific (Folsom, CA) DB-1 (dimethylpolysiloxane, 30 m × 0.53 mm id, 5-μm film). The data were collected and analyzed with a Hewlett Packard (Avondale, PA) 3394 integrator.

Chromatographic Conditions

A 2.0 μL splitless injection was used with the split vent opened after 0.70 min. The helium carrier gas flow was 8.0 mL/min with a split vent flow of 192 mL/min to provide a split ratio of 25:1. The injection port temperature was 200°C, and the flame ionization detector temperature was 275°C. The column oven temperature was programmed by initially holding the temperature at 100°C for 5 min, then increasing to 200°C at a rate of 7°C/min, and finally increasing to 240°C at a rate of 25°C/min, where it was held for 30 min.

Reagents

LC grade 1-butanol was used as the solvent for sample and standard preparation. PCA (Fisher Scientific, Pittsburgh, PA; 98% purity) was used to prepare standards and samples for spike recovery. The CHG-containing and non-CHG-containing alcohol foam surgical scrub products were prepared by the Calgon Vestal Laboratories Skin Care Product Development Group (St. Louis, MO).

Standard Preparation

A PCA standard stock solution was prepared by dissolving approximately 0.01 g of PCA in approximately 10 g of 1-butanol to produce an approximately 1000 ppm PCA stock solution. The exact concentration of the stock solution was calculated, and then appropriate amounts of the stock solution were diluted with 1-butanol to prepare various PCA standards.

Sample Preparation

The following procedure was used to prepare PCA-spiked CHG-containing alcohol foam samples. Approximately 5 mL

of 1-butanol was used to dissolve approximately 1 g of foam and precipitate the CHG. This solution was then syringe-filtered with a 0.45-μm Gelman Acrodisc LC13 PVDF filter to remove the CHG. Approximately 1 g of the filtered solution was then diluted with 4 mL of 1-butanol and divided into 2 aliquots. A known amount of a solution of PCA in 1-butanol was then added to 1 aliquot. The resultant solutions were syringe-filtered to remove any remaining CHG and then analyzed by the GC system. The CHG-containing foam samples were analyzed with and without PCA addition so that the amount of PCA originally in the foam sample could be determined.

The following procedure was used to prepare low-level (containing PCA at ≤2 ppm) spiked placebo foam samples. Unlike the previously described spiked-sample preparation, these placebo foam samples did not contain any CHG or PCA before they were spiked with PCA. A stock solution of PCA in 1-butanol (≤2 ppm) was prepared. Approximately 5 mL of this solution was then used to dissolve approximately 1 g of foam. Because no CHG was present in the placebo foam, no precipitation occurred upon addition of the 1-butanol solution, and the resultant solution did not require filtration. Approximately 1 g of this solution was then diluted with approximately 4 mL of the stock solution of PCA in 1-butanol. The resultant solution was then syringe-filtered (even though it was still clear) and analyzed by the GC system.

Results and Discussion

The mean PCA peak height of at least 2 injections of all standards, samples, and spiked samples were used in the calculations.

Table 1. Summary of data for PCA calibration curve

Run	Number of standards	Correlation coefficient	Slope	y Intercept
1	6	0.9995	15490	−3877
2	6	0.9997	17600	−7375
3	9	0.9997	15100	−2889

Calibration Curve and Data for Limits of Quantitation and Detection

Three calibration curves were generated to assess the linearity of the method. The Run 1 calibration curve was derived from standards containing PCA in the 0.956–10.0 ppm range. The Run 2 calibration curve was derived from standards containing PCA in the 1.06–10.3 ppm range. The Run 3 calibration curve was derived from standards containing PCA in the 0.801–20.0 ppm range. Linearity was found over each standard range, and a summary of these data is found in Table 1.

The limits of quantitation and detection were each determined experimentally by preparing and then analyzing low-concentration PCA standards. The limit of quantitation for PCA was 0.566 ppm. This was the lowest concentration standard that had a peak height that could be measured by the integrator. When standards of lower concentration were prepared and analyzed, a PCA peak was seen, but the integrator did not provide peak height data. The limit of detection was defined as the PCA concentration that produced a signal-to-noise ratio greater than 3. A 0.207 ppm PCA standard was found to be the limit of detection based upon this criterion.

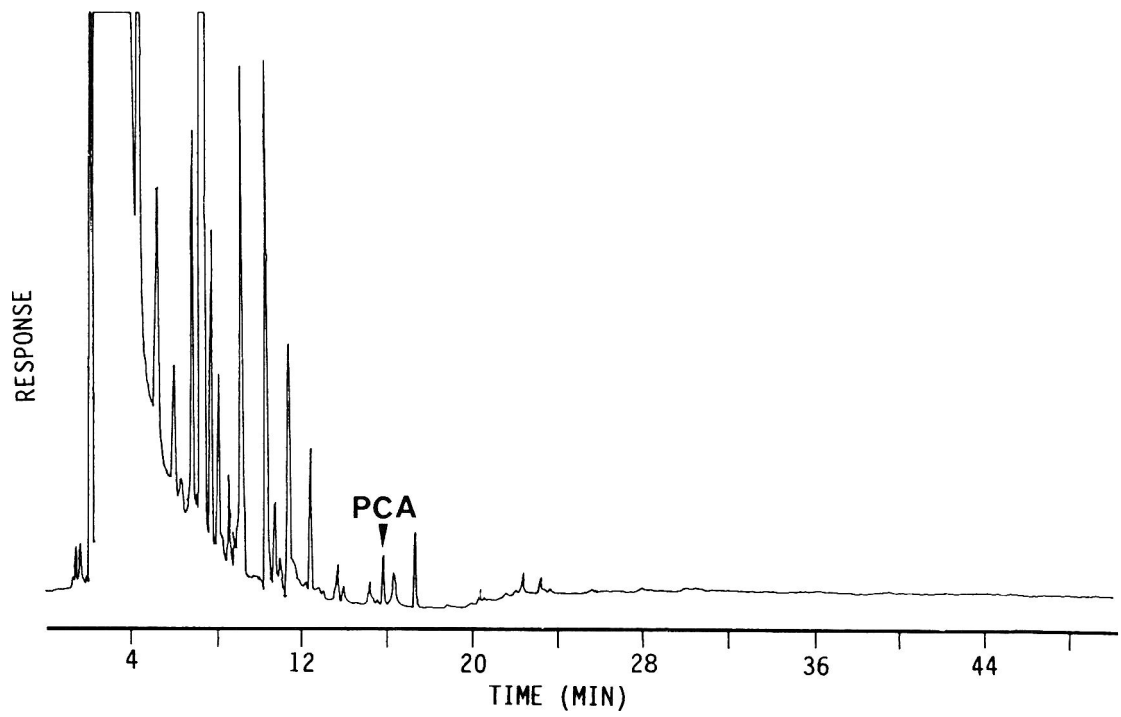

Figure 1. Chromatogram of a 1.14 ppm PCA standard.

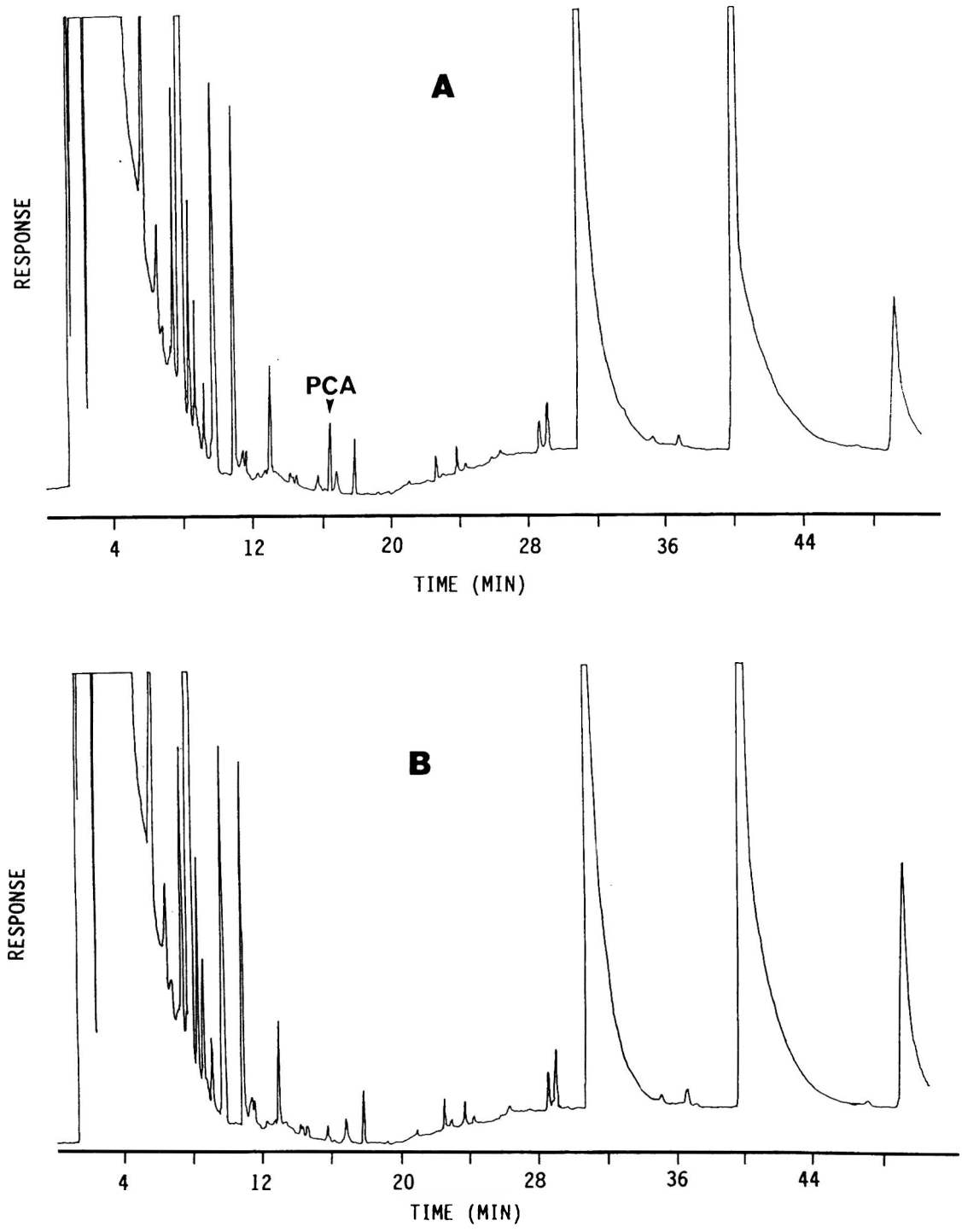

Figure 2. (A) CHG-containing alcohol foam surgical scrub product with PCA at 2.20 ppm. (B) Placebo alcohol foam surgical scrub product; no PCA present.

Precision

To determine the method precision, multiple injections were made of PCA standards and a CHG-containing alcohol foam sample, and then relative standard deviations (RSDs) of the peak heights were calculated. A 5.16 ppm PCA standard injected 6 times gave an RSD of 0.7%. A 5.19 ppm PCA standard injected 10 times gave an RSD of 0.4%. A CHG-containing alcohol foam sample containing PCA at 1.14 ppm PCA and injected 6 times gave an RSD of 1.5%.

Accuracy

To determine the accuracy of the method, both placebo and CHG-containing PCA-spiked foam samples were prepared and analyzed by the GC system, and percent recovery values

were calculated. The initial work involved analyzing a CHG-containing alcohol foam sample for its PCA content and then adding a known amount of PCA and analyzing the sample again. A spiked-sample recovery value was determined by comparing the additional PCA found experimentally with the amount that was known to have been added. Two runs of this type were performed. In Run 1, 3 spiked samples containing PCA in the 3.70–7.22 ppm range were prepared and analyzed, and a mean recovery of 102 ± 1% was found. In Run 2, 4 spiked samples containing PCA in the 3.73–6.64 ppm range were prepared and analyzed, and a mean recovery of 99.5 ± 3% was found. The overall recovery for the 2 runs was 101 ± 3%.

The spiked-sample recovery experiments using the actual CHG-containing foam product were important because they demonstrated that 1-butanol precipitation was effective in removing CHG from the sample matrix. If the CHG was not adequately removed, then high spike recovery values would have been found, because CHG would have degraded in the injection port and produced additional PCA. This type of degradation was observed in early method development work when methanol, in which CHG is soluble, was used as the solvent.

Although spiking a CHG-containing alcohol foam product was effective in demonstrating that the method worked in removing CHG and generating accurate PCA data in that environment, samples containing low levels of PCA (≤2 ppm) could not be evaluated properly for accuracy, because all of the CHG-containing alcohol foam product already contained some amount of PCA. When a placebo (non-CHG-containing) alcohol foam became available, the PCA concentrations of ≤2 ppm could be investigated. Because the method's main utility is in detecting low ppm amounts of PCA, the availability of a true placebo alcohol foam made it possible to perform the necessary recovery experiments for samples spiked with PCA at low levels.

For the first spike recovery run, the concentration range investigated was similar to that used in the spiking experiment for CHG-containing alcohol foam product. Four placebo samples containing PCA in the 4.76–7.22 ppm range were prepared and analyzed, and a mean recovery of 98.5 ± 0.5% was found. Two runs of the placebo samples spiked at low levels were then performed. In the first low-level run, 5 spiked placebo samples containing PCA in the 1.01–1.99 ppm range were prepared and analyzed. The mean recovery was 98.1 ± 1.8%. In the second low-level run, 5 spiked placebo samples containing PCA in the 0.536–2.01 ppm range were prepared and analyzed. The 0.536 ppm sample was detected, but the integrator could not provide peak height data so it was not quantitated. The mean recovery for the remaining 4 samples was 96.9 ± 1.5%. The mean recovery for all 3 runs of the spiked samples of placebo foam was 97.8±1.5%.

The spiked-sample recovery data demonstrate that the method can accurately quantitate PCA when CHG is present in the foam sample and when PCA is present at low part-per-million levels.

Selectivity

A typical *p*-chloroaniline standard chromatogram is shown in Figure 1. Because the chromatogram contains many peaks, determination of the PCA peak was accomplished by comparing a chromatogram of the solvent, 1-butanol, with the chromatogram of the PCA standard. On the basis of this comparison, PCA was found to elute at approximately 16 min. Figure 2 shows a typical chromatogram of a sample of CHG-containing alcohol foam product. Additional peaks found in this chromatogram are due to the foam vehicle. Comparison of this chromatogram with a chromatogram of the placebo foam in Figure 2 (containing no CHG and no PCA) shows that the 16.4 min peak does not appear in the placebo. Because no other peaks occur at this retention time, the data indicate that the method is selective for PCA.

In summary, a GC method has been developed for the analysis of PCA in CHG-containing alcohol foam surgical scrub products, which cannot be analyzed by a reversed-phase LC system. A simple sample preparation with 1-butanol was developed to dissolve the foam and then precipitate the CHG to remove potential interferences that would result from CHG degradation.

Acknowledgment

We thank L. Walker for his help with sample preparation during preliminary method development.

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Niacin II: Identification of Isonicotinic Acid in Niacin by Liquid Chromatography with Diode Array Detection

ROSS D. KIRCHHOEFER
U.S. Food and Drug Administration, Center for Drug Evaluation and Research, Division of Drug Analysis, 1114 Market St, Room 1002, St. Louis, MO 63101

Isonicotinic acid impurity in bulk niacin was detected and identified by comparison with a reference material by liquid chromatography with a diode array detector. The niacin was dissolved in dilute hydrochloric acid and chromatographed on an amine column with a mobile phase of methanol and water acidified with formic acid. Isonicotinic acid has a relative retention time of 1.5 compared with niacin (nicotinic acid), and the wavelengths of maximum ultraviolet (UV) absorbance for isonicotinic acid and niacin are 270 and 260 nm, respectively. The amount of impurity found in the niacin sample was 0.3%. Twelve formulations, including sustained-release products, one bulk material, and the United States Pharmacopeia (USP) reference standard were tested. The impurity was detected in only the bulk and USP reference material samples.

This laboratory was assigned the task of evaluating niacin and niacin products when a report to the U.S. Food and Drug Administration (FDA) indicated that sustained-release niacin formulations, administered to patients for cholesterol control, may cause liver problems (private communication). A sustained-release niacin sample of clinical interest was submitted to this laboratory for investigation.

Separate laboratory investigations into dissolution profiles of tablets, possible impurities in bulk niacin and niacin products, and toxicological testing of components found present in niacin were started.

Liquid chromatography (LC) was chosen as the initial approach to detect and identify impurities present in niacin or niacin products. LC coupled with diode array detection is a powerful tool to detect and identify impurities. Many LC methods have been proposed for the analysis of niacin and related substances, including vitamins in a pharmaceutical (1), liquid tonic (2), and other formulations (3–7); pyridine and niacin-related compounds (8–11); isonicotinic acid in urine (12); and tryptophan and its metabolites (13). LC methods for the analysis of vitamins in foods have been reviewed (14). A variety of LC techniques have been investigated, including diode array detection of nitrogen-containing compounds and vitamins (15, 16), new column packings for vitamin analysis (17), and ion-pairing factors on vitamin separations (18). Scott (19) discussed the degradation of nicotinic acid and related compounds.

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An LC procedure was needed that retained niacin on a column sufficiently to allow examination of both faster and slower eluting compounds. In most of the references cited, the retention time of niacin was not increased to accentuate the resolution of structurally related compounds.

Different columns, including columns with C_8, C_{18}, and CN packings, and different mobile phases, including PIC A and PIC B ion-pairing reagents, were tested. Under these chromatographic conditions, niacin elutes rapidly, precluding the determination of impurities in niacin.

The procedure described in this paper retains niacin sufficiently to allow examination of the chromatogram for impurities. The procedure uses an amine (NH2) column packing and a mobile phase of methanol–acidified water (82 + 18). The water is acidified with formic acid to pH 2–3. The niacin sample is prepared in dilute hydrochloric acid for the analysis. This combination of column and mobile phase provides excellent separation of niacin and isonicotinic acid. The diode array UV spectrum of the impurity as it eluted from the column matched the spectrum of isonicotinic acid reference material chromatographed under identical conditions. The amount of isonicotinic acid found in the niacin bulk sample was 0.3%. Twelve niacin formulations, including sustained-release niacin, the sample of clinical interest, one bulk material, and the United States Pharmacopeia (USP) reference standard were examined. Isonicotinic acid was detected only in the bulk sample and the USP reference standard by this procedure.

The bulk material has not been linked to the sample of clinical interest. Isonicotinic acid has not been linked with liver damage in patients.

Experimental

Reagents

(a) *Solvent.*—Methanol, Optima (Fisher Scientific Co., St. Louis, MO).

(b) *Formic acid.*—99% solution, lot 127F3427 (Sigma Chemical Co., St. Louis, MO).

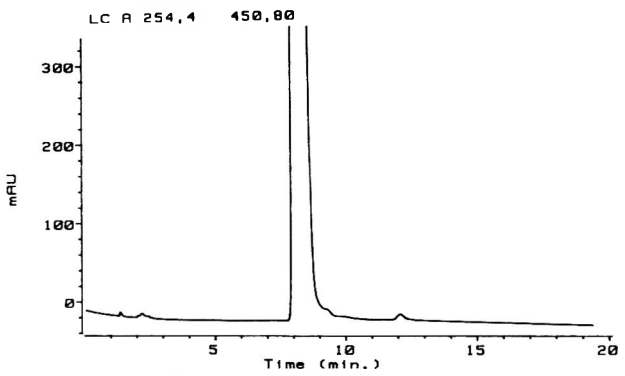

Figure 1. Chromatogram of a solution of bulk niacin showing an impurity component, identified as isonicotinic acid, eluting at about 12 min.

(c) *Water.*—For LC, Waters Milli Q System (Millipore Corp., Bedford, MA).

(d) *Hydrochloric acid.*—Reagent grade ACS; prepare a 0.1 N solution (Taychemco, St. Louis, MO).

(e) *Mobile phase.*—5.0 mL of formic acid is added to 1 L of water; 18 parts of the acidified water is mixed with 82 parts of methanol. Each solvent is filtered thru 0.45 µm filter disks with vacuum.

(f) *Niacin (nicotinic acid).*—Lot BNA 8712001 (Wockhardt Ltd., Gujaret, India).

(g) *Isonicotinic acid.*—Lot 6219 (Eastman Organic Chemicals, Rochester, NY).

(h) *Picolinic acid.*—Lot 45F3408 (Sigma Chemical Co.).

(i) *USP reference standard.*—Lot G-2 (United States Pharmacopeia, Rockville, MD).

Apparatus

(a) *LC column.*—Phenomenex Hypersil 3 NH2, 150 mm × 4.6 mm, 3 µm particle size, part No. 00F-0147-EO (Phenomenex, Torrance, CA).

(b) *Liquid chromatograph.*—Waters 600E system with controller and pump, Wisp 712 autoinjector, flow set to 1.0 mL (Millipore Corp.).

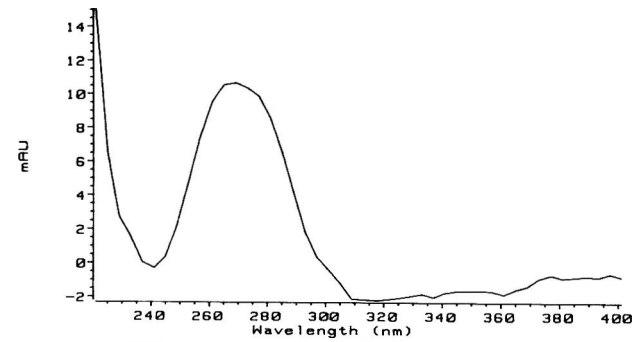

Figure 2. Diode array, full-scan UV spectrum of impurity component, eluting at 12 min, with a UV maximum at 270 nm.

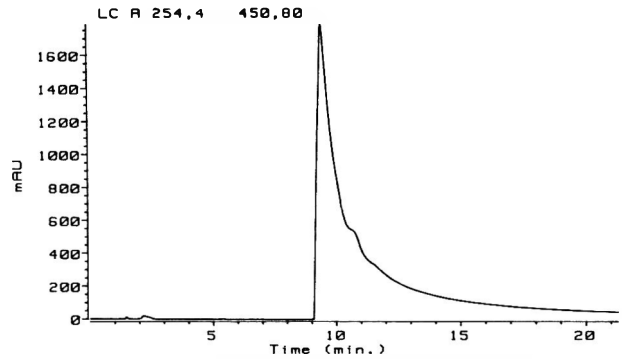

Figure 3. Chromatogram of a solution of picolinic acid.

(c) *Detector.*—HP 1040M diode array detector with HP 300 hard disk drive, HP Thinkjet printer, HP ColorPro plotter, HP Monitor and keyboard with Chemstation software (Hewlett-Packard Co., Palo Alto, CA).

(d) *Membrane filter.*—13 mm, 0.45 µm filter disks held in Swinnex adapter (Millipore Corp.).

Procedure

(a) *Niacin, low concentration.*—Dissolve 5 mg of niacin, accurately weighed, in 10 mL of 0.1 N HCl. This solution is used to set niacin retention time. Inject 25 µL.

(b) *Niacin, high concentration.*—Dissolve 50 mg of niacin, accurately weighed, in 10 mL of 0.1 N HCl (10 times the analytical concentration). Inject 25 µL.

(c) *Mixed standard.*—Dissolve 5 mg each of isonicotinic acid and niacin, accurately weighed, in 10 mL of 0.1 N HCl. Inject 25 µL.

(d) *Picolinic acid.*—Prepare solutions as in (a) and (b) above.

(e) *Tablets.*—Place a portion of a well-mixed, ground composite equivalent to 250 mg of niacin in a 50 mL volumetric flask. Add 25 mL of 0.1 N HCL and sonicate the resulting solution for 30 min with frequent swirling. Dilute to volume with 0.1 N HCL. Filter the solution through a membrane filter; discard the initial filtrate. Inject 25 µL.

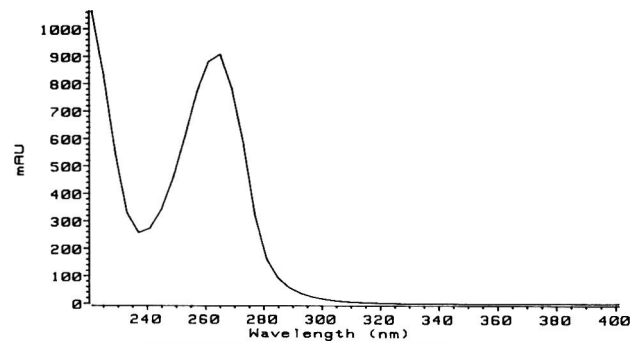

Figure 4. Diode array, full-scan UV spectrum of picolinic acid, eluting about 10 Min, with a UV maximum at 262 nm.

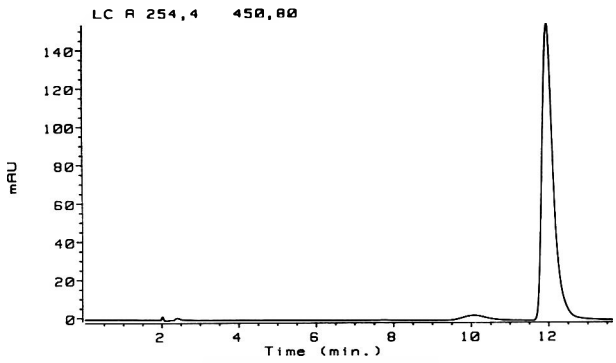

Figure 5. Chromatogram of a solution of isonicotinic acid reference material, eluting at 12 min.

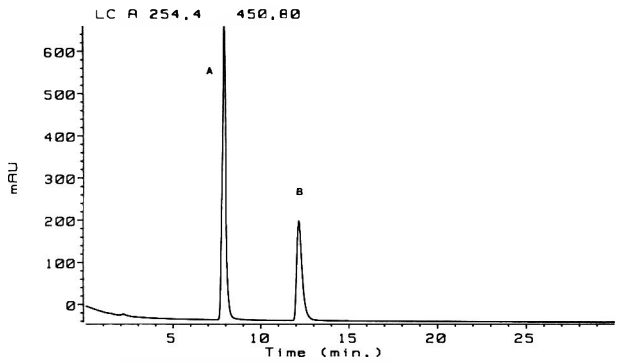

Figure 7. Chromatogram of a solution of niacin (A) and isonicotoninic acid (B).

(f) *Detection.*—Monitor all samples with the diode array at 254 ± 4 nm with a reference of 450 ± 80 nm to produce chromatograms.

Results and Discussion

The procedure used to detect impurities is based on the technique called HI–LO chromatography as discussed by Inman and Tenbarge (20). An injection of a solution with a low concentration (LO) is used to accurately measure the area of the major ingredient. A concentrated solution (HI) of 10–100 times the concentration of the major ingredient is injected to measure any minor (impurity) peaks that might be present. Sufficient resolution must exist between the active ingredient peak and the impurity peak so that the area of the active ingredient in the HI injection does not mask an adjacent impurity peak.

The 10 times injection of the bulk niacin material indicated the presence of an impurity at a relative retention time of 1.5 compared with niacin and another impurity in the tail of the large niacin component. Figure 1 shows the chromatogram obtained from the bulk niacin material with an impurity eluting at 12 min. Figure 2 shows the diode array, full-scan UV spectrum of the impurity peak at 12 min. The UV absorbance maximum of this impurity component is at 270 nm. The following compounds were screened as possible impurity components: quinolinic acid, 3-hydroxyanthranilic acid, kynurenic acid, quinaldic acid, 2-pyridylacetic acid hydrochloride, *dl*-kynurenine, tryptophan, 3-hydroxykynurenine, and xanthurenic acid. On the basis of chromatographic retention and UV absorbance spectra, all of these compounds were eliminated as the observed impurity.

The positional isomers of niacin (nicotinic acid) were investigated. The positional isomers of niacin and picolinic and isonicotinic acids are resolved from niacin with this chromatographic system. Picolinic acid, prepared according to the preparation for niacin [(**a**) in *Procedure*], initially was not detected. At higher levels, at about a 10-fold increase in concentration, picolinic acid was observed. The picolinic acid elutes at about 10 min and exhibits considerable tailing (Figure 3). The UV spectrum of picolinic acid, presented in Figure 4, is similar to the UV spectrum of niacin.

An isonicotinic acid solution was prepared similar to niacin [(**a**) in *Procedure*] and analyzed. Figure 5 shows the resulting chromatogram. The retention time is about 12 min or 1.5 relative to niacin. The UV spectrum is given in Figure 6. The retention time and UV absorbance spectrum of isonicotinic acid match the characteristics of the observed impurity.

A mixed standard solution containing niacin and isonicotinic acid was prepared and injected, and the UV absorbance spectra were recorded. The chromatogram is shown in Figure 7. The UV spectra of niacin and isonicotinic acid are overlaid in Figure 8. Finally, the UV spectrum of isonicotinic acid (Figure 8) was overlaid with the diode array UV spectrum of

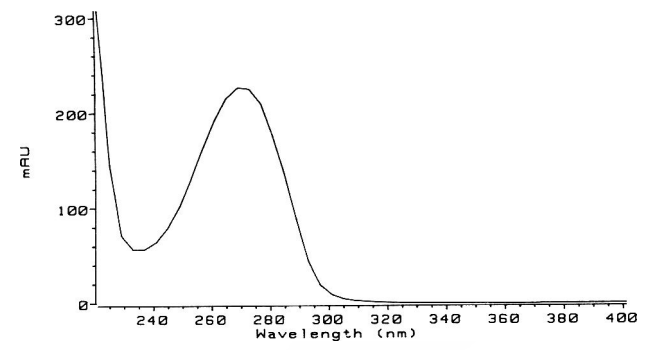

Figure 6. Diode array, full-scan UV spectrum of isonicotinic acid reference material with a UV maximum at 270 nm.

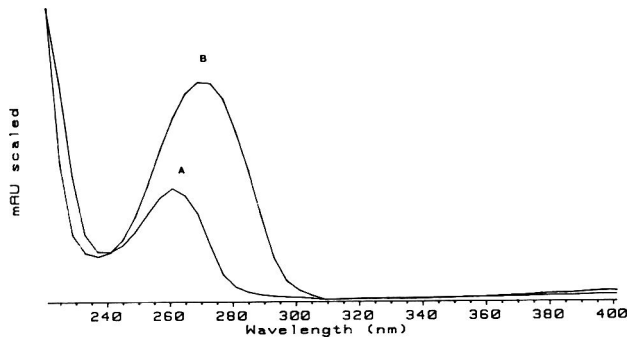

Figure 8. Overlaid diode array, full-scan UV spectra of niacin (A, 260 nm) and isonicotinic acid (B, 270 nm).

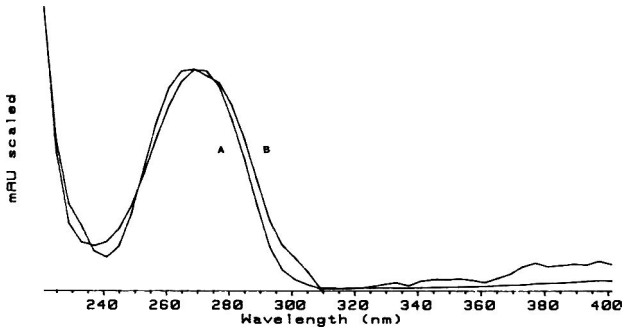

Figure 9. Overlaid diode array, full-scan UV spectra of (A) the impurity at 12 min retention time (Figure 2) and (B) isonicotinic acid reference material (Figure 8).

the impurity (Figure 2) and the results are shown in Figure 9. The overlaid spectra of isonicotinic acid reference material and the impurity peak match, indicating that isonicotinic acid is the impurity detected in the niacin sample.

The amount of isonicotinic acid in the sample was calculated to be 0.3% by using isonicotinic acid as the external standard and area percent. A recovery of isonicotinic acid spiked in niacin at the 1% level (1/100) gave a recovery of 103%. The limit of detection with the procedure as described is about 0.08%.

Examination of 12 niacin formulations (including the sample of clinical interest), one bulk material, and the USP reference material showed only the bulk material and the USP reference material to have isonicotinic acid present. The amount of isonicotinic acid found in the USP reference material was slightly less, about 0.1–0.2%.

It was also noted that the retention time of niacin drifts to slightly faster retention times with each subsequent injection.

The use of 0.1 N acetic acid as a sample solvent stops the retention time drift.

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DRUGS, COSMETICS, FORENSIC SCIENCES

Determination of Nitrofuran Residues in Poultry Muscle Tissues and Eggs by Liquid Chromatography

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A liquid chromatographic (LC) method was developed and statistically validated for the determination of nitrofurazone, furazolidone, and furaltadone residues in poultry and porcine muscle tissues. The antimicrobial residues were extracted with a mixture of dichloromethane and ethyl acetate by using ultrasonication followed by solid-phase extraction cleanup and LC analysis with UV detection. A modification of the method incorporating acetonitrile extraction and solvent partition cleanup was developed for analysis of poultry eggs. The limits of detection were 1 μg/kg for nitrofurazone and furazolidone and 2 μg/kg for furaltadone in both muscle tissues and eggs. Average recoveries for spike levels of 1, 2, and 5 μg/kg ranged from 84 to 128%, and coefficients of variation were between 1.1 and 12.1%. A field trial with these methods was conducted in conjunction with the Western Australian Department of Agriculture to determine the stability of furaltadone in both poultry tissue and eggs. Results of this study show that the concentration of furaltadone in muscle tissue diminished rapidly even when stored at −18°C. Furaltadone was considerably more stable in eggs.

Nitrofurans are used in veterinary medicine, alone or in combination with other drugs, to promote growth or improve feed efficiency. They are administered either orally or topically in prophylactic and therapeutic treatment of mainly *Escherichia coli* and *Salmonella* infections in calves, pigs, and poultry and as coccidiostats in poultry (1). Nitrofurans are metabolized extensively, with most of the absorption of metabolites occurring through intestinal mucosa (2).

Nitrofurans are mutagenic and (pro)carcinogenic (2). Consequently, their use has been strictly regulated in many countries, and tolerance levels of 1–2 μg/kg have been set for the parent nitrofurans. As part of the National Residue Survey (NRS) administered by the Australian Government Department of Primary Industries and Energy, the Australian Government Analytical Laboratories (AGAL) were asked to develop a sensitive method for the simultaneous determination of residues of nitrofurazone (5-nitro-2-furaldehyde semicarbazone), furazolidone (*N*-[5-nitro-2-furfurylidene]-3-amino-2-oxazolidone), and furaltadone (3-[5-nitro-2-furfurylideneamino]-5-[4-morpholinomethyl]-2-oxazolidone) in plasma and muscle tissues. Their structures are depicted in Figure 1. The NRS program aims to ensure compliance with maximum residue limits (MRLs) (3). The MRL for furazolidone is 0.01 mg/kg in edible offal (mammalian) and muscle tissues. No MRL has yet been established for nitrofurazone or furaltadone.

Nitrofurans have a characteristic UV absorption, which was used in a number of analytical procedures to enable sensitive detection. Initially, we followed a method to analyze nitrofurans in plasma that was provided through private communications from the National Chemical Residue Analytical Laboratory of the New Zealand Ministry of Agriculture and Fisheries in New Zealand. This method was based on a previously published procedure (4) and used a commercially available disposable diatomaceous earth solid-phase extraction (SPE) column. Although specified recoveries were achieved for plasma at a spike level of 5 μg/kg, interfering peaks were experienced at lower levels, predominantly from the SPE column. Several modifications were investigated without success; therefore, we decided to deviate from this approach in favor of a general procedure that uses an ultrasonication solvent extraction system, which we use for other antibiotic residue analyses at AGAL.

This report describes the development of a simple method for the routine analysis of nitrofuran residues in muscle tissue and eggs at the tolerance levels specified. During development of the method, emphasis was placed on obtaining a clean final extract, with full resolution of the nitrofurans of interest and minimal interference from sample coextractives, while, at the same time, achieving acceptable residue recovery. The report also discusses the stability of furaltadone residues in poultry muscle and egg samples obtained from a field trial.

Experimental

The method was validated by using spiked muscle tissue and egg, as well as field samples containing incurred residues of furaltadone. The incurred samples were obtained from feed trials undertaken in conjunction with the Western Australian

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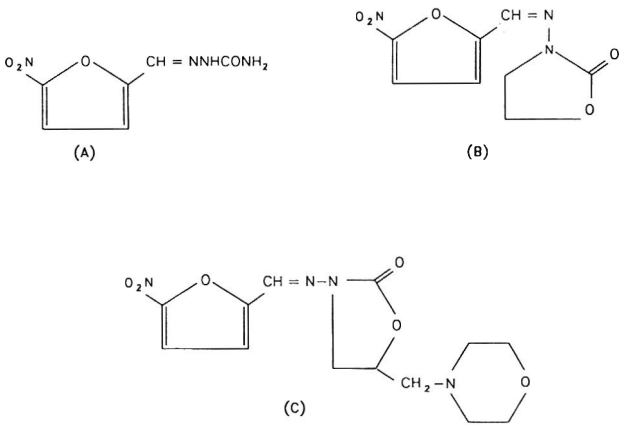

Figure 1. Structures of (A) nitrofurazone, (B) furazolidone, and (C) furaltadone.

Department of Agriculture using a veterinary preparation (C.C.D. Furaltadone) in the drinking water of hens.

A number of hens were maintained for 6 days on medicated water containing 107 mg/L furaltadone, after which some of the hens were slaughtered (day 1 kill). Medication was then ceased for the remainder of the hens by transferring them to nonmedicated water. A number of these remaining hens were then slaughtered on day 2 and day 3.

All muscle samples were initially analyzed on the day of slaughter. To assess the stability of furaltadone residues, muscle samples from day 1 kill, which contained significant furaltadone residues, were reanalyzed after 2, 4, and 32 days of storage at −18°C in the absence of light.

Egg samples were collected for 4 days commencing from the day before slaughter (day 0). Each day's collection was combined and homogenized to provide 4 samples. Each of the prepared samples was stored in a deep freeze at −18°C, in the absence of light, pending analysis. The eggs were analyzed after 56 days of storage and reanalyzed after 76 and 92 days of storage.

Apparatus

(a) *Domestic blender.*—Breville wizz-kid.

(b) *Domestic mincer.*—Kenwood blender mixer, Model A516.

(c) *Ultrasonic bath with timer.*—Soniclean 500T.

(d) *Rotary evaporator.*—Corning rotary evaporator, Type 349/2, connected to water vacuum; water bath temperature, 40–50°C.

(e) *Evaporation manifold.*—Pierce Reacti-Vap, Model 8780, operated with air at room temperature.

(f) *Centrifuge.*—Beckman GP series with GH-3.7 rotor.

(g) *Liquid chromatograph.*—Waters Associates (Milford, MA) Model 590 programmable LC pump, Model 486 tunable absorbance detector operated at 362 nm, Model 712 WISP autosampler, 25 cm × 4.6 mm id, 5 µm, Spherisorb ODS-2 column (Alltech Associates, Deerfield, IL). The liquid chromatograph was operated isocratically at 1.1 mL/min under ambient temperature and 1700 psi; attenuation, 0.005 AUFS.

(h) *Guard column.*—10 mm × 4.6 mm id, 5 µm, packed with Spherisorb ODS-2.

(i) *Macro-pipettor.*—Oxford or similar, with disposable plastic tips.

Reagents

(a) *Solvents.*—LC grade acetonitrile, dichloromethane, and methanol (Ajax Chemical Co., Sydney, Australia); dimethyl formamide, analar grade (BDH Chemicals, Victoria, Australia); ethyl acetate, analar grade (BDH Chemicals); and petroleum ether (Shell X_4), freshly redistilled in glass; water, further purified by using a Barnstead NANOpure II (United States) system to obtain type I water.

(b) *Mobile phase.*—Acetonitrile–water (1 + 3). Degas before use.

(c) *Sodium sulfate.*—Granular, anhydrous, AR grade. Heat at 160°C for a minimum of 16 h before use.

(d) *Silica Sep-Pak.*—Waters No. 51900.

(e) *Acrodisc LC13PVDF 0.45µ* filters.—Gelman or similar.

(f) Reference standards.—Curator of Standards, AGAL, Sydney, Australia.

(g) *Individual stock solutions.*—1.0 mg/mL. Dissolve 25 mg furazolidone and 25 mg furaltadone each into 20 mL acetonitrile in 25 mL volumetric flask, and dilute to volume with acetonitrile. Dissolve 25 mg nitrofurazone into 20 mL dimethyl formamide in 25 mL volumetric flask, and dilute to volume with dimethyl formamide. Prepare fresh every 2 months.

(h) *Intermediate composite standard.*—10 µg/mL. Dilute 1 mL of each of the nitrofuran stock solutions to 100 mL with acetonitrile in a 100 mL volumetric flask. Renew weekly.

(i) *LC calibration standard.*—100 ng/mL. Dilute 1 mL of the intermediate composite standard to 100 mL with mobile phase in a 100 mL volumetric flask. Renew weekly.

(j) *Seeding standard for recovery studies.*—50 ng/mL. Dilute 1 mL of the intermediate composite standard to 100 mL with ethyl acetate in a 100 mL volumetric flask. Further dilute this solution by pipetting 25 mL into a 50 mL volumetric flask, and dilute to volume with ethyl acetate. Renew weekly.

Note: All reference standards must be covered with aluminum foil to protect them from both fluorescent and direct sunlight and must be stored at −18°C. All other prepared standard solutions must be covered with aluminum foil and stored at 4°C when not in use. All preparation and manipulation steps for standard solutions were conducted in artificial yellow light (4).

Sample Preparation

During sample preparation and at all subsequent stages of analysis, samples must be protected from exposure to both fluorescent light and direct sunlight. All operations on samples for nitrofuran analysis in this study were carried out in artificial yellow light (4).

Muscle tissue was prepared for analysis by trimming excess fat and then mincing the tissue. Eggs were deshelled and blended (not beaten) and then stored frozen at −18°C in the

absence of light, until analysis could be performed. Prior to analysis, egg samples were thawed to room temperature in the dark and mixed thoroughly.

Sample Extraction

Muscle tissue.—Weigh a 10 g sample of minced muscle tissue, and mix the tissue with ca 10 g sodium sulfate in a 250 mL glass centrifuge bottle. Add 40 mL dichloromethane–ethyl acetate (5 + 3). Sonicate for 10 min, and centrifuge for 5 min at 1600 rpm. Filter supernatant through a plug of silanized glass wool in a funnel into a 250 mL flat-bottom, round flask. Re-extract the sediment twice more. Combine extracts, and evaporate them to near dryness on a rotary evaporator at 45°C. Add 30 mL dichloromethane to the flask, and evaporate the mixture to dryness. Redissolve the residues in 5 mL dichloromethane, and then add 5 mL petroleum ether.

Eggs.—Weigh a 10 g sample of blended egg, and mix the egg with ca 10 g sodium sulfate in a 250 mL glass centrifuge bottle. Add 40 mL acetonitrile. Sonicate for 10 min and centrifuge for 5 min at 1600 rpm. Transfer the supernatant by using an Oxford macro-pipettor with disposable plastic tip into a 250 mL flat-bottom, round flask. Re-extract the sediment with 2 additional 30 mL portions of acetonitrile. Combine all extracts, and rotary evaporate them at 40°C to less than 10 mL volume. Transfer the concentrated extract into a 50 mL centrifuge tube with an additional 5 mL acetonitrile. Add 15 mL acetonitrile-saturated petroleum ether and mix thoroughly. Centrifuge the mixture for 5 min at 2800 rpm. Discard the upper petroleum ether layer. Transfer the acetonitrile layer into a 100 mL pear-shaped flask. Add 20 mL petroleum ether. Rotavap this solution to near dryness at 40°C to ensure complete removal of acetonitrile. Add a further 10 mL petroleum ether, and again rotary evaporate the solution to dryness. Redissolve the sample extract in 5 mL dichloromethane, and then add 5 mL petroleum ether.

SPE Cleanup

Attach a silica Sep-Pak (SPE column) to a 20 mL Luer-lok syringe, and prewet with 2 mL petroleum ether. Care should be taken not to let the Sep-Pak cartridge run dry, because this may result in lower recoveries. Load the sample extract followed by flask washings with 4 mL dichloromethane–petroleum ether (1 + 1). Maintain the flow rate through the Sep-Pak at ca 2 drops/s by applying positive pressure at the inlet. Wash the Sep-Pak with 5 mL petroleum ether, fit it onto the evaporation manifold, and let it dry for 15 min under a steady stream of air. Elute the Sep-Pak with 15 mL methanol followed by 10 mL ethyl acetate–methanol (1 + 1) into a 50 mL pear-shaped flask. Rotary evaporate the eluate just to dryness at 45°C, and remove the last traces of solvent by blowing with a gentle stream of nitrogen.

Quantitation

Redissolve the nitrofuran residues in 0.5 mL of mobile phase, and filter through a 0.45 µm membrane filter into an LC vial. Analyze the LC Calibration Standard and sample extracts by injecting 40 µL of each into the LC. All samples with residue concentrations exceeding the linear calibration range should be diluted appropriately and reinjected on the same day of analysis.

Results and Discussion

The method obtained from New Zealand for the analysis of nitrofuran in plasma was found to give satisfactory results at higher concentrations (5 µg/kg) but suffered from interferences when used at lower concentrations. Modification of the extraction procedure, using a previously published procedure for the analysis of furazolidone in pig muscle and plasma (5), enabled us to achieve consistent recoveries on spiked plasma samples with minimal interference problems. However, when we attempted to use this methodology to analyze muscle tissues, problems were encountered because the final extract was fatty, resulting in interference in the LC analysis. A literature survey revealed a number of published analytical methods for simultaneous determination of nitrofuran residues in tissues using SPE with alumina column cleanup (6–8). We investigated the use of both alumina and silica for SPE cleanup and found silica provided a more effective cleanup for the particular sample matrixes, residue types, and levels encountered in our work.

To achieve optimum recoveries for nitrofurazone and furaltadone in muscle tissue, a mixture of dichloromethane and ethyl acetate was used as the extracting solvent. However, this solvent was not suitable for the extraction of nitrofuran residues in eggs, owing to the formation of insoluble proteinaceous material which blocked the SPE cartridge during the cleanup step. Deproteination with acetonitrile, as recommended by Carignan et al. (5), gave an extract free of insoluble material from the egg matrix.

A further problem was encountered with the analysis of eggs because they have a high fat content compared to poultry muscle tissue, which interferes with both SPE cleanup and final LC analysis. Therefore, it was necessary to remove excess fat prior to the SPE cleanup. This was achieved by partitioning the egg extract with acetonitrile-saturated petroleum ether.

Extraction efficiency tests were conducted on an egg sample containing naturally incurred furaltadone residue to compare ultrasonication with homogenization techniques (Table 1). The results indicate comparable extraction efficiency. The quantity of muscle tissue with incurred residue was limited at the time of these trials. For this reason, comparative extraction efficiency testing was not conducted on tissue in this work.

Table 1. Results of replicate analyses[a] of an egg sample containing naturally incurred furaltadone residue using ultrasonication and homogenization extraction techniques

Result	Ultrasonication	Homogenization
Furaltadone, µg/kg	81	80
$SD^{(n-1)}$	6.7	4.7
RSD, %	8.2	5.9

[a] ($n = 5$)

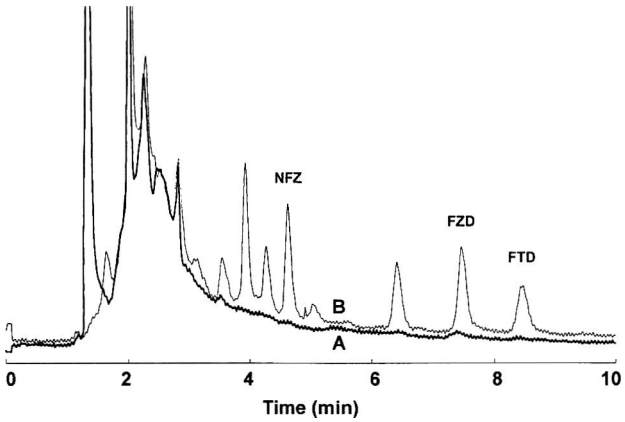

Figure 2. Typical chromatograms of (A) blank and (B) a spiked poultry muscle tissue with 5 μg/kg each of nitrofurazone (NFZ), furazolidone (FZD), and furaltadone (FTD).

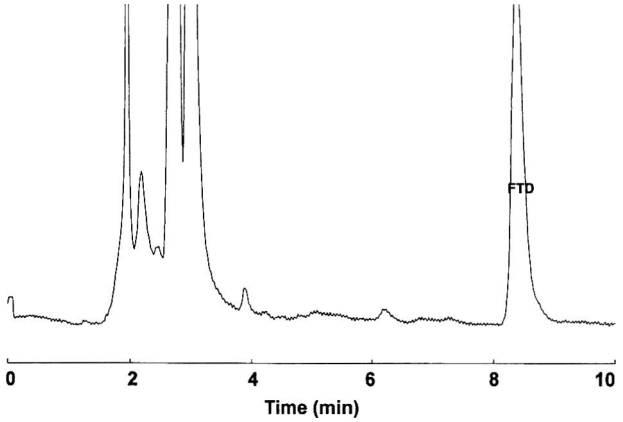

Figure 4. Typical chromatogram of an egg sample with furaltadone (FTD) from hens fed with C.C.D. Furaltadone.

However, previous experience at AGAL has shown extraction efficiency in muscle tissue using these 2 techniques to be comparable to other antimicrobial analyses. We preferred to use ultrasonication, because it was faster and there was less potential for interference from coextractives.

The effectiveness of the cleanup procedures described permitted chromatographic analysis under isocratic conditions, and chromatograms obtained for both tissue and egg samples were free of extraneous peaks. Figures 2 and 3 illustrate the typical chromatograms of a spiked poultry muscle and a spiked egg sample along with appropriate sample blanks. In Figure 4, a typical chromatogram of an egg sample from the hens fed with C.C.D. Furaltadone is shown.

The effectiveness of residue recovery was established by replicate analyses of muscle and egg samples spiked with nitrofuran standards at 1, 2, and 5 μg/kg (Table 2). The mean recoveries (84–111%) for both sample types are acceptable, with the exception of furaltadone, in muscle tissue spiked at 2 μg/kg (128%). This higher recovery was caused by the presence of low level interference. The sensitivity of the method, expressed as 2 times the peak-to-peak noise, was 0.5 μg/kg for nitrofurazone and furazolidone and 1 μg/kg for furaltadone. Regression analysis of the data obtained by injecting a series of calibration solutions in the range 0.1–1.0 μg/mL showed the detector response to be linear ($R^2 > 0.999$).

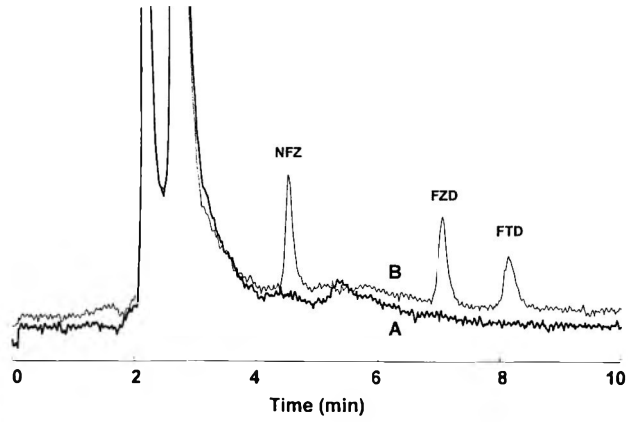

Figure 3. Typical chromatograms of (A) blank and (B) a spiked egg sample with 5 μg/kg each of nitrofurazone (NFZ), furazolidone (FZD), and furaltadone (FTD).

Field Trial Results

Results from the field trial samples are summarized in Table 3. Muscle samples were analyzed on the day of slaughter, whereas eggs were stored for 56 days prior to analysis, as discussed in the *Experimental* section.

Significant concentrations of furaltadone were found in muscle tissue samples from day 1 kill, but none was detected in muscle samples from day 2 or day 3 kill hens. However, significant concentrations of furaltadone were detected in all egg samples collected over the 4 day period. In a literature report of the results of similar trials with furazolidone (9), the parent compound was detected in eggs laid up to 9 days after withdrawal of medicated feed. Our results show a similar trend for furaltadone, with residues being detected in eggs laid at least

Table 2. Recovery of nitrofuran residues from spiked poultry muscle tissues and spiked poultry eggs

		Muscle		Eggs	
Residues	Spike level, μg/kg	Mean rec., %[a]	RSD, %	Mean rec., %[a]	RSD, %
Nitrofurazone	1	86	8.6	99	12.1
	5	84	4.2	87	5.2
Furazolidone	1	109	7.3	111	10.3
	5	86	3.7	85	5.7
Furaltadone	2	128	11.8	97	8.7
	5	95	1.1	87	5.7

[a] Mean of 5 determinations.

Table 3. Concentration of furaltadone in muscle tissue and eggs of hens fed with C.C.D. Furaltadone

	Furaltadone, μg/kg	
	Muscle	Eggs[a]
Day 0	NA[b]	190, 232
Day 1 kill	362 ± 18.0[c]	115, 129
Day 2 kill	ND[d]	91 ± 10.5[c]
Day 3 kill	ND	42, 38

[a] Analyzed after 56 days of storage at −18°C in absence of light.
[b] NA = not applicable.
[c] Mean of 5 determinations.
[d] ND = not detected.

4 days after withdrawal of medicated water. Owing to the delay in analysis of egg samples, the relationship between levels of furaltadone detected in the eggs after storage and original levels present in the samples when first taken (on each day of kill) has not been established.

To establish the stability of furaltadone in muscle tissue stored at −18°C and in the absence of light, progressive analysis of day 1 kill muscle was conducted. The analysis results listed in Table 4 show a rapid decay of furaltadone in muscle tissue even when the tissue is stored under these conditions. After 96 h of storage, the level of furaltadone dropped to less than 25% of the original residue level. Similar results were observed in earlier published field trials using furazolidone (5). The observed results in the storage stability trial were more variable than anticipated. One possible reason for this is that, although muscle samples were thoroughly homogenized prior to analysis on day 1 kill, they were not rehomogenized prior to subsequent reanalyses. The furaltadone decay may not have been uniform throughout the frozen stored muscle sample, thus resulting in some variability. The progressive analysis results for eggs stored at −18°C and in the absence of light are summarized in Table 5. In general, there is a decrease in furaltadone concentration as the period of storage increases, although the overall rate of furaltadone decay appears to be somewhat less in eggs than in muscle tissue.

The results of this study indicate that samples should be analyzed as soon as possible after receipt at the laboratory, since even under controlled storage conditions, depletion of residues occurs relatively quickly. This is an important consideration for AGAL and similar residue monitoring laboratories, where analytical results need to accurately reflect levels of residues present in foodstuff.

Table 4. Stability of furaltadone in muscle tissue from hens fed with C.C.D. Furaltadone

Storage time, days	Furaltadone, μg/kg	$SD^{(n-1)}$	RSD, %
0	362[a]	18.0	5.1
2	189, 97	NA[b]	NA
4	76[c]	13.4	17.7
32	50[a]	18.7	37.2

[a] Mean of 5 determinations.
[b] NA = not applicable.
[c] Mean of 4 determinations.

Table 5. Stability of furaltadone in eggs from hens fed with C.C.D. Furaltadone (duplicate analysis)

Storage time, days	Furaltadone found, μg/kg			
	Day 0	Day 1 kill	Day 2 kill	Day 3 kill
56	190, 232	115, 129	91[a]	42, 38
76	156, 162	81, 99	NT[b]	36, 38
92	159, 131	108, 101	NT	32, 35

[a] Mean of 5 determinations; RSD = 10.5.
[b] NT = not tested.

Acknowledgments

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Gas Chromatographic Determination of Chloramphenicol Residues in Shrimp: Interlaboratory Study

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An interlaboratory study of a gas chromatographic method for determining chloramphenicol (CAP) residues in shrimp was conducted. An internal standard (I_{std}), the meta isomer of CAP, was added to the shrimp, and the treated shrimp were homogenized with ethyl acetate. The ethyl acetate extract was defatted with hexane, and the CAP was partitioned into ethyl acetate from an aqueous salt solution. The ethyl acetate was evaporated, and the dried residue was treated with Sylon, a trimethylsilyl derivatizing agent, to yield the trimethylsilyl derivative of CAP. A portion of the solution containing the derivative was injected into a gas chromatograph equipped with an electron capture detector. Levels of fortified and incurred CAP were calculated from the peak area ratio of standard CAP to I_{std}. Recoveries of CAP from tissue directly fortified at 5 ppb were 102% (within-laboratory relative standard deviation [RSD_r] = 5.6%), 104% (RSD_r = 5.5%), and 108% (RSD_r = 6.3%) from Laboratories 1, 2, and 3, respectively. Incurred-CAP residues at 5 and 10 ppb levels were also determined, with the following results: Laboratory 1: composite A, 4.56 ppb (RSD_r = 14.0%); composite B, 8.38 ppb (RSD_r = 11.6%); Laboratory 2: composite A, 4.17 ppb (RSD_r = 12.5%); composite B, 8.90 ppb (RSD_r = 5.60%); Laboratory 3: composite A, 4.66 ppb (RSD_r = 14.9%); composite B, 11.0 ppb (RSD_r = 11.8%).

Chloramphenicol (CAP), a broad-spectrum antibiotic that was developed around 1950, has very effective antibacterial properties. Several years of clinical use have produced a significant amount of evidence relative to the serious toxic effects of CAP on humans (1). There is the potential for misuse in domestic and international food market products (1, 2), because CAP is effective in animal therapy, including the treatment of aquaculture species. CAP residues may be present in the edible portion of treated animals and thereby pose a health risk to consumers. The U.S. Food and Drug Administration (FDA) banned its use in treating food-producing animals.

World shrimp farming has grown 6-fold in a single decade, with the harvest estimated at 565 000 tons in 1989 (2). Competitive pressure associated with this kind of growth has intensified farming practices, which make disease control a primary concern. Because CAP is a very effective antibiotic, it is often used prophylactically for disease control. This use could result in the occurrence of CAP residues in farmed commercial shrimp (2). Thus, a sensitive analytical method that monitors CAP residues in farm-raised shrimp is needed.

CAP is readily soluble in most polar solvents but only slightly soluble in water. Its relative water insolubility is the basis of several published methods that have, as part of their cleanup, a partition step extracting CAP from water into ethyl acetate (3–6; personal communication, Food Safety and Inspection Service [FSIS], U.S. Department of Agriculture [USDA], with modifications by Allen, Jacobson, and Wiseman, FDA, 1982). Other methods partition CAP onto a lipophilic solid-phase column from aqueous solutions (7, 8). Liquid chromatographic separation with UV detection is the most often used analytical technique (8–13) for determining CAP, but it lacks sensitivity in the low parts-per-billion (<50 ppb) range. The measurement of trace levels of CAP requires a more sensitive gas chromatographic (GC) method using electron capture detection (ECD) (6, 7; personal communication, FSIS, USDA, with modifications by Allen, Jacobson, and Wiseman, FDA, 1982). Therefore, the proposed method was developed for determining CAP in shrimp at the 5–10 ppb range by using GC/ECD. The 3 FDA laboratories that participated in the validation of this method were the Seattle and Denver Districts and the Animal Drugs Research Center.

METHOD

Apparatus

(a) *Gas chromatographs.*—Hewlett-Packard Model 5880 with packed column and Model 5890 with splitless injector for

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capillary columns (Hewlett-Packard Co., Avondale, PA), both equipped with ^{63}Ni electron capture detector. Temperatures for packed column oven, detector, and injector were set at 270°C isothermal, 340°C, and 290°C, respectively. For capillary columns, oven programs were 150°C to 270°C at 20°C/min, held 8.50 min, and 270°C to 290°C at 20°C/min, held 2 min, with injection port and detector temperatures set at 270°C and 300°C, respectively.

(**b**) *Columns.*—(*1*) Glass 6 ft × 4 mm id, packed with 3% OV-7 on 100/120 mesh Supelcoport (No. 1-1788M; Supelco, Inc., Bellefonte, PA); carrier gas, methane–argon (5 + 95), 30 mL/min. (*2*) HP-5 fused-silica capillary column, cross-linked 5% phenyl methyl silicone film, 0.33 μm thickness, 25 m × 0.2 mm id (Hewlett-Packard); carrier gas, He, ca 1 mL/min.

(**c**) *Rotary evaporator.*—Buchi R-110 with ice trap (Brinkmann Instruments, Inc., Westbury, NY).

(**d**) *Evaporator.*—N-Evap Model 111 (Organomation Associates, Inc., South Berlin, MA).

(**e**) *Syringe.*—10 μL, Series 700 (Hamilton Co., Reno, NV).

(**f**) *Flask.*—Pear-shaped, 100 mL, 24/40 ⅁ (No. 608700-0224; Kontes Co., Vineland, NJ).

(**g**) *Tubes.*—Falcon graduated 50 mL centrifuge tubes (No. 2098; Becton Dickinson, Lincoln Park, NJ); disposable polypropylene tube, 13 × 100 mm; glass, 13 and 50 mL graduated centrifuge tubes with glass stopper (No. 410550-013 and 411650-0000; Kontes Co.).

(**h**) *Pasteur pipet.*—Disposable 5.75 in. (14.6 cm).

(**i**) *Centrifuge.*—Model 6000 with Model 823A rotor (International Equipment Co., Needham Heights, MA).

(**j**) *Aspirator.*—500 mL vacuum flask with 20 × ⅛ in. od × 1/16 in. id Teflon tube fitted through rubber stopper with side arm of flask connected to water aspirator vacuum.

(**k**) *Mixer.*—Vortex-Genie Model No. S8223 (Scientific Products Div., Baxter Co., McGaw Park, IL).

(**l**) *Homogenizer.*—Tissuemizer Model SDT 1810 with Model 18N shaft (Tekmar Co., Cincinnati, OH).

(**m**) *Blender.*—5-speed pulsed Oster Model 54841 (Baxter Co.).

(**n**) *Pipettor.*—Adjustable 5 mL (No. 851350; Wheaton Manufacturers, Millville, NJ); microadjustable 20–200 μL (No. P-200; Rainin Instrument Co., Woburn, MA).

Reagents

(**a**) *Solvents.*—Distilled-in-glass, pesticide-grade, nonspectra-grade ethyl acetate, hexane, and toluene (Burdick & Jackson Laboratories, Inc., Muskegon, MI); USP absolute ethanol (US Industrial Chemical Co., New York, NY).

(**b**) *Sodium chloride.*—ACS grade. Prepare 4% aqueous solution.

(**c**) *Trimethylsilyl derivatizing agent.*—Sylon HTP kit, 1 mL ampules (Supelco, Inc.).

(**d**) *CAP standards.*—USP chloramphenicol (U.S. Pharmacopeia, Rockville, MD). (*1*) *Stock solution.*—Weigh 10.0 mg CAP standard into 100 mL volumetric flask, and dilute to volume with MeOH (100 μg/mL). (*2*) *Working solution (Std).*—Pipet 1.00 mL CAP stock solution into 100 mL volumetric flask, and dilute to volume with MeOH (1000 ng/mL).

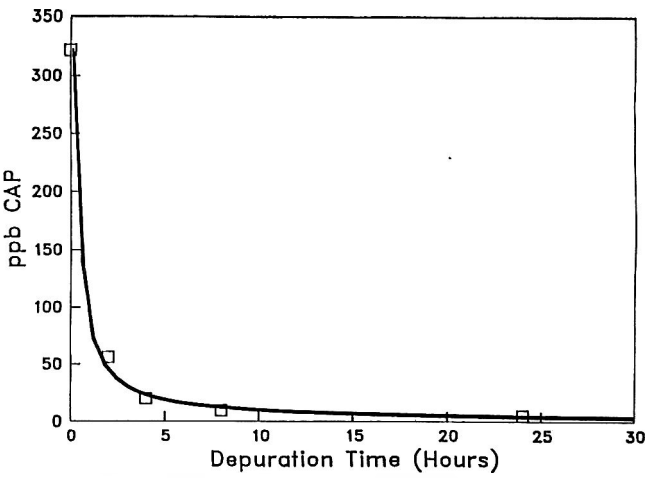

Figure 1. Depletion of chloramphenicol from water-depurated shrimp over a 24 h period. Shrimp were treated with 25 ppm CAP for 4 h before transfer to clean water. This curve represents the CAP residue remaining in tissue during a 24 h period.

(**e**) *Internal standards.*—*m*-Nitrochloramphenicol (M-CAP) (synthesized at University of Georgia, School of Chemical Science, Department of Chemistry, Athens, GA). (*1*) *Stock solution.*—Weigh 10.0 mg M-CAP into 100 mL volumetric flask, and dilute to volume with MeOH. (*2*) *Working solution (I_{std}).*—Pipet 1.00 mL aliquot of M-CAP stock solution into 100 mL volumetric flask, and dilute to volume with MeOH (1000 ng/mL).

(**f**) *Derivatized standards solution (D_{mix}).*—Pipet 100 μL aliquot from both Std and I_{std} working solutions into 15 mL centrifuge tube, and dry under steady flow of N_2. Derivatize as described under derivatization section, except make final volume to 1.00 mL.

Homogenization, Extraction, and Cleanup

Hold frozen shrimp at room temperature until they feel limber. Remove heads, chitinous shell, and body appendages from thawed shrimp. Place shrimp meat in blender, and blend with pulsed action until contents are uniform.

Table 1. Recovery of chloramphenicol[a] from shrimp fortified at various levels

		Recovery		
Added, ppb	Found, ppb	$\bar{X}$, %	SD, %	RSD_r, %
0	0	—	—	—
1.21	1.30	107	4.8	4.5
2.42	2.48	102	4.6	4.5
4.86	4.96	102	5.7	5.6
9.72	10.2	105	4.8	4.6

[a] $n = 5$.

Table 2. Relative recovery of chloramphenicol from fortified shrimp in interlaboratory study

	Laboratory 1			Laboratory 2			Laboratory 3		
Test portion	Added, ppb	Found, ppb	Relative rec., %	Added, ppb	Found, ppb	Relative rec., %	Added, ppb	Found, ppb	Relative rec., %
				Control shrimp					
1	0	0	—	0	0.0	—	0	0	—
2	0	0	—	0	1.36	—	0	0	—
3	0	0	—	0	0.80	—	0	0	—
4	0	0	—	0	0.57	—	0	0	—
5	0	0	—	0	0.0	—	0	0	—
6	—	—	—	0	0.0[a]	—	—	—	—
7	—	—	—	0	0.0[a]	—	—	—	—
8	—	—	—	0	0.0[a]	—	—	—	—
9	—	—	—	0	Trace[a]	—	—	—	—
				Foritified shrimp					
1	4.86	4.49	92.4	5.26	5.15	97.9	5.05	5.88	116
2	4.86	5.00	103	5.26	5.45	104	5.05	5.40	107
3	4.86	5.05	104	5.26	5.92	112	5.05	5.68	112
4	4.86	5.35	110	5.26	5.51	105	5.05	4.98	98.6
5	4.86	4.91	101	5.26	5.20	98.9	5.05	5.31	105
6	4.86	4.96	102	—	—	—	—	—	—
$\bar{X}$			102			104			108
SD			5.7			5.6			6.8
RSD_r, %			5.6			5.5			6.3

[a] Repeat test of control composite.

Weigh 10 g blended shrimp composite into 50 mL polypropylene centrifuge tube, add 100 µL I_{std} and 20 mL ethyl acetate to tissue, and prewash homogenizer blade by briefly blending small volume of ethyl acetate. Discard solvent. Homogenize for 10–15 s at medium speed. Centrifuge at 3000 rpm for 5 min, and decant solvent containing extract into 100 mL pear-shaped flask. Homogenize solids with additional 20 mL ethyl acetate, centrifuge homogenate at 3000 rpm for 5 min, and decant solvent containing extract into flask containing first extract. In a rotary evaporator, evaporate combined extract to 2–4 mL in water bath set at 50°C, and completely transfer to 50 mL glass centrifuge tube with Pasteur pipet. Wash pear-shaped flask with three 2 mL portions of ethyl acetate, swirl flask after each wash, and add washes to tube. Place tube in N-Evap with water bath set at 50°C. Completely evaporate solvent under steady flow of N_2. Add 25 mL 4% aqueous NaCl solution, 2 mL methanol, and 15 mL hexane to tube containing dry residue. Stopper tube and shake vigorously for 1 min. Centrifuge tube for 1 min at 800 rpm. Remove upper hexane layer by aspiration. Repeat extraction step with 2 additional 15 mL portions of hexane; discard hexane each time. Extract CAP from aqueous phase by shaking for 30 s with 15 mL ethyl acetate. Centrifuge mixture for 1 min at 800 rpm. Carefully remove upper solvent layer containing CAP with pipettor and transfer layer to clean 100 mL pear-shaped flask. Repeat extraction with additional 15 mL portion of ethyl acetate and combine extracts. In a rotary evaporator, evaporate combined extracts at 55°C to 2–4 mL. Completely transfer extract to 15 mL centrifuge tube by rinsing flask with three 2 mL portions of ethyl acetate. Evaporate to dryness under steady flow of N_2 in water bath set at 50°C. Rinse walls of tube with 1 mL absolute ethanol. Continue evaporation until tube contents are dry. Proceed to derivatization step.

Derivatization

Add 100 µL Sylon to residue in centrifuge tube, stopper tube, and mix (Vortex Genie) to moisten walls of tube. Place tube in water bath set at 55°C for 40 min. Evaporate contents carefully to *near* dryness, maintaining appearance of wetness, with stream of N_2 at room temperature. Add 500 µL toluene and mix (Vortex Genie) contents of tube. (The final volume can be increased with toluene for test portions containing >5 ppb CAP.) Inject 2–3 µL aliquot into gas chromatograph.

Determination

Inject aliquot of D_{mix} before and after each set of 5 test solutions. [*Note*: Bracketing with D_{mix} maintains a continuous check of detector response.] Determine peak area ratio (CAP/I_{std}) for each test solution. In calculations, use average peak area ratio obtained for D_{mix} injected before and after each set of 5 test solutions. Calculate concentration of CAP residue in test portion (ng/g) as follows:

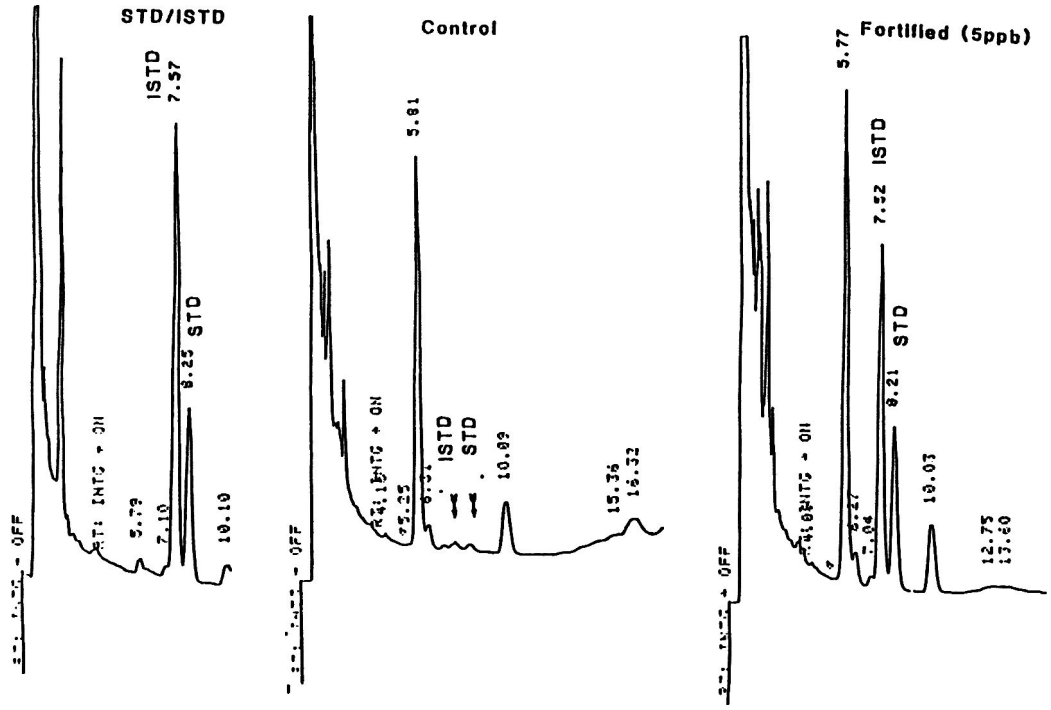

Figure 2. Gas chromatograms from determination of chloramphenicol in shrimp on a packed OV-7 column.

$$\text{CAP, ng/g} = X \times C \times V/Y \times 1/W$$

where X = peak area ratio (CAP/I_{std}) for test solution, C = concentration of Std in D_{mix} (100 ng/mL), V = final volume of test solution (mL), Y = average peak area ratio for D_{mix} injections, and W = weight of test portion (10 g).

Interlaboratory Study

Preliminary Test

Analysts were asked to perform a preliminary performance test before proceeding with the study itself. A preliminary test was designed to test the ability of the participants to recover CAP at a level of 5 ppb by using this procedure. Minimum acceptable recovery of CAP was set at 60% for this test. Five portions of one set of control shrimp were weighed and fortified with CAP and M-CAP at 5 and 10 ppb, respectively. A second set of 5 portions of control shrimp were fortified only with 10 ppb M-CAP internal standard. Both sets were analyzed by the method described. The analyses of the shrimp fortified with M-CAP only were used to demonstrate the repeatability of the procedure. A mixture of CAP and M-CAP working solutions was also prepared at the same concentrations and derivatized. This derivatized mixture (D_{mix}) was injected into the gas chromatograph before and after each set of 5 test solutions from analysis of fortified shrimp controls. The average peak area ratio for the D_{mix} injection was used to calculate CAP recoveries.

Table 3. Determination of incurred chloramphenicol (CAP) in shrimp in interlaboratory study

	CAP found, ppb		
Composite	Laboratory 1	Laboratory 2	Laboratory 3
A[a]	4.40	4.38	5.81
	5.51	5.58	4.63
	4.18	5.05	4.35
	4.14	4.17	4.59
	Lost	4.37	3.94
$\bar{X}$	4.56	4.17	4.66
SD	0.64	0.59	0.70
RSD_r, %	14.0	12.5	14.9
B[b]	7.78	9.39	9.96
	9.98	8.67	10.9
	8.58	9.19	9.70
	7.59	8.13	11.6
	7.97	9.11	12.9
$\bar{X}$	8.38	8.90	11.0
SD	0.97	0.50	1.3
RSD_r, %	11.6	5.60	11.8

[a] Composite of CAP-incurred shrimp depurated for 8 h; $n = 5$.
[b] Composite of CAP-incurred shrimp depurated for 4 h and control shrimp; $n = 5$.

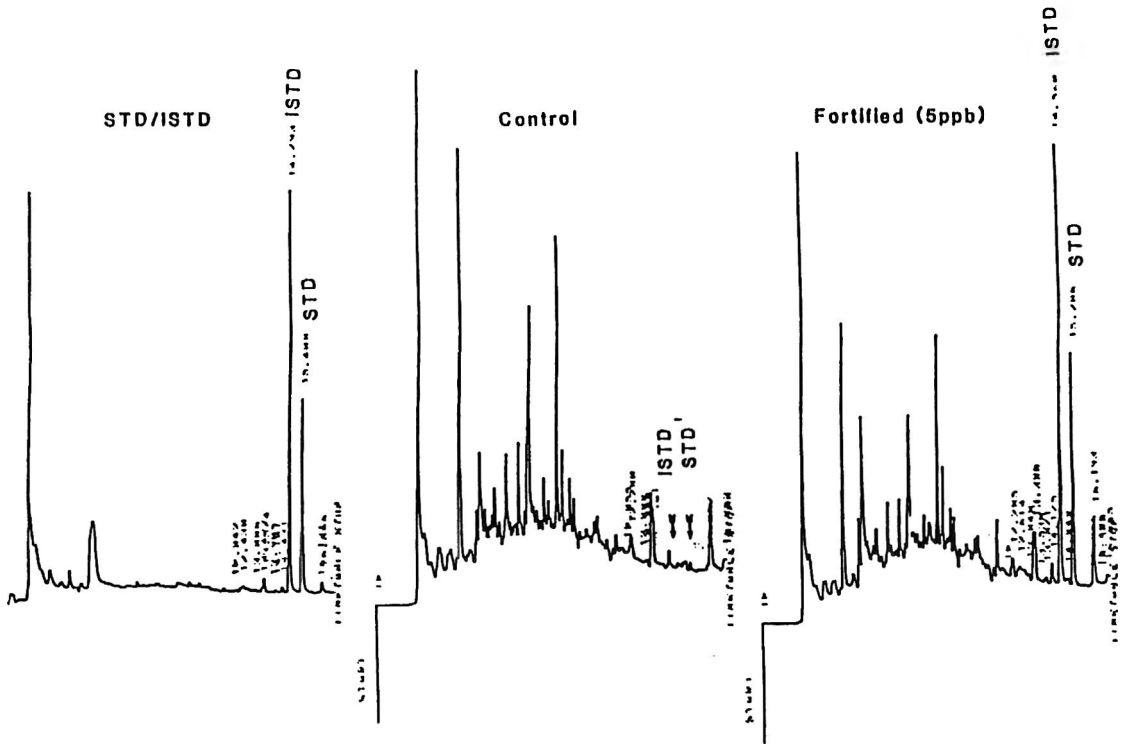

Figure 3. Gas chromatograms from determination of chloramphenicol in shrimp on a 5% phenyl methyl silicone-coated capillary column.

Incursion/Depuration Procedure

Live shrimp (mean body weight = 13 g) were obtained by trawl from the Mississippi Sound, north of Dauphin Island, AL. Shrimp were treated by placing them in water that contained 25 ppm CAP. The shrimp were removed after 4 h, rinsed, and then transferred to clean water (with activated carbon) for depuration. At predetermined intervals, groups of 5 shrimp each were removed from the clean depuration water and sacrificed. Two portions from each depuration group were analyzed for CAP, and the concentrations were plotted versus depuration time (Figure 1). The depuration time required to produce shrimp with the desired levels of incurred CAP was estimated from this calibration curve.

Preparation of CAP-Incurred Shrimp

Two shrimp composites with incurred CAP were used for this test. One composite containing CAP at ≥10 ppb was prepared by thoroughly blending 5 control shrimp with 5 CAP-incurred shrimp that were previously found to contain ≥30 ppb CAP. A second composite containing incurred CAP at 5 ppb was taken directly from the depuration water and homogenized. A set of 5 portions from each of the prepared composites was analyzed by participants.

Results and Discussion

During the development of the method, 4 CAP fortification levels were tested for practicality and repeatability. Table 1 gives recovery data for those 4 levels. All recoveries had acceptable within-laboratory relative standard deviations (RSD_r), but the 5 ppb level seemed to be the best choice, on the basis of the relative sizes of the CAP and M-CAP GC peaks.

The participants met the performance criteria, with absolute recoveries of CAP at the 5 ppb level exceeding 80%. There was good agreement among participants for percentage of CAP recovered from fortified shrimp (Table 2). However, the CAP peak found in the chromatogram for the control shrimp by Laboratory 2 (Table 2) suggested the presence of a contaminant or possible cross-contamination. This contamination was confirmed when Laboratory 2 repeated the procedure and obtained only minor or no peaks at the CAP retention time. Recoveries of CAP tended to be near 100% when CAP/M-CAP peak area ratios were used in the calculations. RSD_r values of 5.6, 5.5, and 6.3% for Laboratories 1, 2, and 3, respectively, indicate that acceptable recoveries can be achieved with this method.

To avoid heterogeneity of the final composite resulting from variation in the size of the CAP-incurred shrimp, each composite was blended and assayed to ensure uniformity. Once homo-

geneity was established, the composites were sent to participants. The composite of CAP-incurred shrimp that were depurated in clean water for 8 h was designated composite A. Composite B, consisting of CAP-incurred shrimp that were depurated 4 h and control shrimp, was mixed to obtain a CAP level of about 10 ppb. The RSD_r values reported (Table 3) for CAP-incurred shrimp were generally higher than the RSD_r values reported (Table 2) for CAP-fortified shrimp; however, all RSD_r values were within permissible limits for drug residue in tissue (14).

Gas chromatograms obtained by using the packed OV-7 column and the capillary 5% phenyl methyl silicone column are shown in Figures 2 and 3, respectively. The packed column gave near-baseline separation of the CAP Std and the M-CAP I_{std}. Separation of the Std peak from the I_{std} peak was superior with the capillary column. There were no interferences that would affect the quantitation of incurred CAP, as shown in the control chromatogram of Figure 2. Chromatograms presented here are typical of those submitted by collaborators. This method appears to be suitable for the determination of CAP in shrimp at the 5 ppb level. Van Ginkel et al. (6) reported a GC/mass spectrometric procedure for the confirmation of CAP identity that could be used in conjunction with the method presented here.

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FOOD BIOLOGICAL CONTAMINANTS

Deoxyribonucleic Acid Hybridization Method for the Detection of *Listeria* in Dairy Products, Seafoods, and Meats: Collaborative Study

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The method is based on the hybridization of synthetic deoxyribonucleic acid probes to ribosomal ribonucleic acid sequences unique to *Listeria*. This method was compared to 2 culture methods: the U.S. Food and Drug Administration method for the detection of *Listeria* in dairy products and seafoods and the U.S. Department of Agriculture, Food Safety and Inspection Service method for *Listeria* in meats. Six food types with replicate samples containing various concentrations of *Listeria* were analyzed by the collaborating laboratories. *Listeria* was detected in 774 samples using the DNAH method and in 772 samples using a culture method. The DNAH and culture methods were in agreement for 668 samples containing *Listeria* and 306 samples without *Listeria*. The overall rate of agreement between methods was 82.3%. The method has been adopted first action by AOAC INTERNATIONAL.

A ready-to-use deoxyribonucleic acid hybridization (DNAH) method for the detection of *Listeria* in foods was first described by Klinger et al. (1). The *Listeria* is first increased by culture enrichment and then is captured by filtration on membrane filters. Captured cells are lysed to release ribosomal ribonucleic acid (rRNA). A synthetic DNA probe complementary in sequence to *Listeria* rRNA and labeled with a phosphorus radioisotope hybridizes to the captured *Listeria* rRNA. Hybridization is monitored with a simple beta particle detector.

An improved DNAH method using liquid-phase hybridization, a unique hybrid capture method, and a colorimetric endpoint detection method was described (2, 3). The bacterial cell suspension is lysed by the addition of enzymatic reagents to release rRNA. A probe solution that contains 2 types of synthetic DNA (the capture probe and the detector probe) is added. Both probes hybridize to *Listeria* rRNA, and permit capture and detection of rRNA. The polydeoxyadenylic acid tail on the capture probe hybridizes to complementary polydeoxythymidylic acid sequences on a plastic dipstick. The plastic dipstick is then transferred to a solution with an antibody-enzyme conjugate that recognizes the detector probe. The enzyme portion of the conjugate cleaves an added chemical substrate and produces a visual color that is quantified using a photometer.

The probes used in the GENE-TRAK assay were designed to detect all *Listeria* and have been tested using 188 strains of *L. monocytogenes*, 40 of *L. innocua*, 19 of *L. welshimeri*, 22 of *L. seeligeri*, 11 of *L. ivanovii*, 3 of *L. grayi*, and 3 of *L. murrayi* (GENE-TRAK Systems, unpublished results). Cross-reaction of these *Listeria* probes with non-listeriae has not been observed (GENE-TRAK Systems, unpublished results).

The enrichment culture procedure for the solid-phase radioisotopic DNAH method made use of a buffered secondary enrichment broth (1). This enrichment followed 24 h incubation in either the FDA *Listeria* enrichment broth (LEB) or the USDA UVM broth and enhanced recovery in the presence of acid-producing organisms. A secondary enrichment is also used for the colorimetric DNAH method. Lithium chloride

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phenylethanol moxalactam agar (LPM) was used initially, and then replaced with modified lithium chloride ceftazidime agar (mLCA), a modification of the LCA agar first described by Lachica (4). A swab dipped into the 24 h primary enrichment is used to inoculate the agar plate. After 24 h, bacteria on the plate are removed with a swab and suspended in buffer for analysis by the DNAH method.

In trials, the DNAH methods have proven to be as effective as the culture methods for the detection of *Listeria* in food samples (2, 3). A pilot study designed for compliance with AOAC requirements was conducted prior to the collaborative study (5). Twenty dairy, seafood, meat, and poultry food samples were analyzed in parallel using either the FDA or the USDA culture method and the DNAH method. For each food sample there were 20 subsample controls, 20 subsamples inoculated with a low level of *Listeria*, and 20 subsamples inoculated with a high level of *Listeria*. Target inoculation levels were 1 cell/25 g test unit for the low level and 10 cells/25 g test unit for the high. A total of 822 inoculated or control subsamples were positive for *Listeria* and 378 inoculated or control subsamples were negative. Overall there were 615 subsamples positive by culture method and 769 subsamples positive by DNAH. Agreement between methods was 78.3%. In addition, 10 naturally contaminated samples were tested 10 times each. Eighty-six subsamples were positive by DNAH method, 84 were positive by culture method, and agreement between them was 92.0%.

Collaborative Study

Design of the Study

Six food items (2% milk, Brie cheese, roast beef, frankfurters, raw ground pork, and cooked crabmeat) commonly tested for *Listeria*, frequently contaminated with *Listeria*, or that have been a source of *Listeria* infection, were selected for the study. The milk was inoculated with a strain of *L. innocua*. The cheese and crabmeat were inoculated with strains of *L. monocytogenes* serotypes 3a and 1/2b, respectively. Roast beef was inoculated with a strain of *L. monocytogenes* 3b and was naturally contaminated. Frankfurters and pork were to be inoculated with *L. monocytogenes* serotypes 4b and 1/2a, respectively. Since samples of these products naturally contaminated with *L. monocytogenes* became available, inoculation was unnecessary.

Collaborating laboratories were sent 15 samples of the food item to be analyzed. Samples were labeled with numbers from 1 to 15, the food type, storage temperature, and the date for analysis. Each sample weighed 60–75 g. The inoculation or contamination status of the numbered test samples was known by the reference laboratory only. The milk, cheese, roast beef, and frankfurters were shipped in containers at 4–7°C the day before analysis. Pork and crab samples were shipped frozen 5 or 6 days before analysis. Recommended storage temperatures conformed with the shipping temperatures.

The collaborators were instructed to analyze each of the samples by both the culture method and the DNAH method (Figure 1). Suspect *Listeria* isolates on selective plates, derived from streaking FDA broths, USDA broths, and positive DNA cell suspensions, were identified to species using the FDA protocol.

Preparation of Test Samples

(**a**) *Milk, 2%.*—One gallon containers. The L. *innocua* isolate used for inoculation was cultured in trypticase soy broth for 24 h at 35°C. The cfu level in the broth was determined by direct microscopic count. The culture was diluted with Butterfield's buffer and then used to inoculate the milk at the 2 inoculation levels 4 days prior to analysis. The milk was stored at 4°C prior to shipment. Target inoculation levels were 0.04 cells/g for the low and 0.4 cells/g for the high.

(**b**) *Brie cheese.*—6 lb wheels, domestic, cut into 60–75 g pieces and placed in Whirl-Pak bags. Samples were inoculated 7 days prior to the date of analysis and stored at 3–4°C. High level *Listeria* sample bags were inoculated with 1 mL of a 10^6 cells/mL suspension and a 10^4 cells/mL suspension was used for the low level bags. The samples were mixed by hand kneading. *Listeria* levels declined 3 to 6 logs in 7 days.

(**c**) *Crabmeat.*—Imported frozen cooked crab, 5 lb blocks. The crab was partially thawed at 4°C overnight and 12 lb was mixed with a Hobart mixer at 4°C. High and low level samples were prepared by adding the *Listeria* inoculum during mixing. Target inoculation levels were 0.04 cells/g for the low and 0.4 cells/g for the high. The test samples were prepared from the mixed preparations and stored frozen for 2 weeks prior to analysis.

(**d**) *Frankfurters.*—1 lb packages with similar code dates for each of 3 brands. Frankfurters of a similar brand were blended together in a Hobart mixer at 4°C. The mixtures for 2 of the brands were determined to contain *L. monocytogenes* and were used without inoculation for the 2 contamination levels. The third mixture did not have detectable levels of *Listeria* and was used as the uninoculated control.

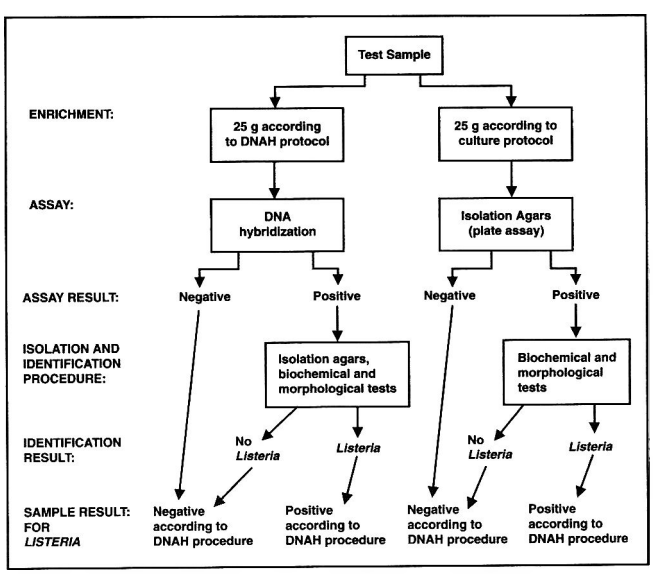

Figure 1. Procedure for analysis of test sample.

Table 993.09 Method performance for detection of *Listeria* in dairy products, seafoods, and meats by colorimetric deoxyribonucleic acid hybridization

Food	*Listeria* level	Method agreement, %	Incidence of false negatives among total positive samples, %		Sensitivity rate		Incidence of false positives among total negative samples, %		Specificity rate	
			DNAH	Culture	DNAH	Culture	DNAH	Culture	DNAH	Culture
2% Milk	high	80.0	15.6	4.7	84.4	95.3	—	—	—	—
	low	76.9	16.4	8.2	83.6	91.8	—	—	—	—
	control	100.0	—[a]	—	—	—	1.6	0.0	98.4	100.0
Brie cheese	high	96.7	1.7	1.7	98.3	98.3	—	—	—	—
	low	86.7	3.4	10.2	96.6	89.8	—	—	—	—
	control	100.0	—	—	—	—	1.7	6.7	98.3	93.3
Crab	high	52.3	14.1	34.4	85.9	65.6	—	—	—	—
	low	47.7	28.6	52.4	71.4	47.6	—	—	—	—
	control	90.8	—	—	—	—	0.0	20.6	100.0	79.4
Frankfurters	high	69.2	30.5	3.4	69.5	96.6	—	—	—	—
	low	56.9	39.7	8.6	60.3	91.4	—	—	—	—
	control	100.0	—	—	—	—	7.7	7.7	92.3	92.3
Roast beef	high	98.7	0.0	1.3	100.0	98.7	—	—	—	—
	medium	89.3	2.7	8.1	97.3	91.9	—	—	—	—
	low	81.3	5.8	14.5	94.2	85.5	—	—	—	—
Ground pork	high	89.2	0.0	10.8	100.0	89.2	—	—	—	—
	medium	87.7	1.6	10.9	98.4	89.1	—	—	—	—
	low	76.9	16.9	8.5	83.1	91.5	—	—	—	—

[a] Undefined.

Table 1. Participation of laboratories in collaborative study

Lab.	Food set						
	2% Milk	Brie cheese	Crab 1	Crab 2	Frankfurters	Roast beef	Raw ground pork
1	Y[a]	Y	Y	Y	Y	Y	Y
2	Y	Y	—	Y	Y	Y	Y[b]
3	Y	Y	Y	Y	Y	Y	Y
4	—[c]	—	Y[d]	—	—	Y	Y[d]
5	Y	Y	Y	Y	Y	Y	Y
6	Y	Y	Y	Y	Y	Y	Y
7	Y	Y	Y	Y	Y	Y	Y
8	Y	Y	Y	Y	Y	Y	Y
9	Y	Y	Y	—	Y	Y	Y
10	Y	Y[d]	Y	Y	Y	Y	Y
11	Y	Y	Y	Y	—	Y	Y
12	Y[d]	—	Y	—	—	—	Y[d]
13	Y	—	Y	Y	Y	Y	Y
14	Y	Y	—	—	Y	Y	Y
15	Y	Y	Y	Y[b]	Y	Y	Y
16	—	Y	—	Y	—	—	—
17	—	—	—	—	Y	Y	Y
18	—	—	—	Y	—	—	—
19	—	—	—	Y	—	—	—
Sample sets	14	13	13	14	13	15	16

[a] Samples sent to collaborator.
[b] Samples analyzed on the wrong date or not at all, results not used.
[c] No samples sent to collaborator.
[d] Sample shipment delayed by carrier or collaborator unable to complete the analyses, no analyses.

Table 2. Results of milk analyses for the presence of *Listeria*[a]

	High inoculum samples					Low inoculum samples					Control samples				
Lab.	3	5	7	8	10	4	9	12	14	15	1	2	6	11	13
						DNAH method									
1	+[b]	+	+	+	+	+	–[c]	–	+	+	–	–	–	–[c]	–
2	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–
3	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–
5	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–
6[d]	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
7	+	+	+	+	+	+	+	+	+	+	–	–	x[e]	–	–
8	+	+	+	–[f]	–[f]	+	+	–[f]	+	–	–	–	–	–	–
9	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–
10	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–
11	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–
13	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–
14	+	+	+	+	+	–	+	+	+	+	–	–	–	–	–
15[d]	–	–	–	–	+	–	–	–	–	+	–	–	–	–	–
						FDA method									
1	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–
2	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–
3	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–
5	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–
6	+	+	+	+	+	+	–	+	–	–	–	–	–	–	–
7	+	+	+	+	+	+	+	+	+	+	–	–	x[e]	–	–
8	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–
9	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–
10	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–
11	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–
13	–[g]	+	+	+	–[g]	+	–[g]	+	+	+	–	–	–	–	–
14	+	–[g]	+	+	+	–	–	–	–	+	–	–	–	–	–
15	+	–[g]	+	+	+	+	+	+	+	+	–	–	–	–	–

[a] Most probable number for the following: high inoculum samples, 0.24/g; low inoculum samples, 0.24/g; and control samples, <0.003/g.
[b] +, *Listeria* detected in sample; –, *Listeria* not detected in sample.
[c] The DNAH assay was positive and the assay confirmation was negative.
[d] Laboratory results were statistical outliers.
[e] No sample.
[f] The DNAH assay was negative, but *Listeria* was recovered from the assay broth.
[g] Selective plates had colonies typical of *Listeria*, but were not *Listeria* on confirmation.

(e) *Roast beef.*— Frozen roast beef with gravy. The meat was cut into small pieces and finely chopped in a food processor. One-third of the meat was inoculated with *Listeria* at a high level and a second third was inoculated with *Listeria* at a low level. The remaining meat was used for the uninoculated control samples. Inoculated samples were mixed thoroughly with a Hobart mixer at 4°C. Target inoculation levels were 0.04 cells/g for the low and 0.4 cells/g for the high. Samples were stored at 4°C for 7 days prior to analysis.

(f) *Raw ground pork.*—Obtained from 3 markets for the high, low, and control sample types. Samples were determined to be naturally contaminated with *Listeria*. The meat from each market was mixed thoroughly in a Hobart mixer at 4°C and then stored frozen for 2 weeks prior to analysis.

Transport

Sets of 15 samples (each weighing 60–75 g) for each food item were sent to collaborators for analysis. Refrigerated foods were sent the day before analysis and the frozen foods were sent 5 or 6 days before analysis. Recommended storage temperatures conformed with the shipping temperatures. All shipments were via overnight shipper. Each sample was supplied in a double Whirl-Pak bag and was labeled with a sample number from 1 to 15, the product type, storage temperature, and date for analysis. The samples were placed in a 1 gallon paint can with refrigerated or frozen ice packs. The can was placed in a Styrofoam shipping container with additional refrigerated or frozen icepacks. Containers were labeled for compliance with

Table 3. Results of brie cheese analyses for the presence of *Listeria*[a]

	High inoculum samples					Low inoculum samples					Control samples				
Lab.	1	2	9	11	15	4	5	8	10	12	3	6	7	13	14
							DNAH method								
1	+[b]	+	+	+	+	+	+	+	+	+	–	–	–	–	–
2	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–
3	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–
5	+	+	–	+	+	+	+	+	+	+	–	–	–	–	–
6	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–
7	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–
8	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–
9	+	+	+	+	+	+	+	+	+	+	–	–[c]	–	–	–
11	+	+	+	+	+	+	–[c]	+	+	+	–	–	–	–	–
14[d]	–[c]	+	+	+	+	+	–[c]	+	–[c]	+	–	–	–	–	–
15	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–
16	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–
							FDA method								
1	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–
2	+	+	+	+	+	+	+	+	–	+	–	–	–	–	–
3	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–
5	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–
6	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–
7	+	+	+	+	+	+	+	–	+	+	–	–	–	–	–
8	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–
9	+	+	+	+	+	+	+	–[e]	+	+	–	–[e]	–[e]	–[e]	–[e]
11	+	+	+	+	+	–	+	+	–	+	–	–	–	–	–
14	–[e]	–[e]	+	+	+	+	+	+	–[e]	–[e]	–	–	–	–	–
15	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–
16	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–

[a] Most probable number for the following: high inoculum samples, 11/g; low inoculum samples, 1.5/g; and control samples, <0.003/g.
[b] +, *Listeria* detected in sample; –, *Listeria* not detected in sample.
[c] The DNAH assay was positive and the assay confirmation was negative.
[d] Laboratory results were statistical outliers.
[e] Selective plates had colonies typical of *Listeria*, but were not *Listeria* on confirmation.

the Dangerous Goods Regulations of the International Air Transport Association (6) and requirements of the specific carrier.

Analysis of Samples

Two enrichments (5 g each) were prepared. One was prepared according to the recommended reference method and the second as specified by the DNAH method. The reference method for the milk, crab, and cheese samples was the FDA culture method of Lovett and Hitchins (7). The reference method for meats was the USDA culture procedure (8). Bacterial colonies on isolation media for each of the methods—DNAH, FDA, and USDA—which exhibited the *Listeria* morphology were identified using the Lovett and Hitchins (7) protocols.

The level of *Listeria* in samples was determined by most probable number (MPN) techniques by the reference laboratory on the same day samples were analyzed by the collaborators. Triplicate samples of 100, 10, 1, and 0.1 g were analyzed according to the appropriate method, FDA or USDA, using 900, 90, 9, and 10 mL of enrichment broth, respectively. For some foods, MPN/g results were the same for 2 inoculation levels even though the inoculation level differed by 10-fold. In these instances, the level refers to inoculum level.

Statistical Calculations

Results were studied using the outlier test recommended by McClure (9) for qualitative methods. The outlier test was applied to DNAH and culture method results. Inoculated and naturally contaminated samples were classified as positive samples. Control samples without *Listeria* were classified as negative. Results identified as outliers for either method were not eliminated for reasons presented under "Results and Discussion."

Table 4. Results of crabmeat analyses for the presence of *Listeria*[a]

	High inoculum samples					Low inoculum samples					Control samples				
Lab.	4	5	9	10	15	2	6	8	11	12	1	3	7	13	14
DNAH method using an enrichment protocol not recommended for seafoods															
1	–[b]	–	–	–	–	–	–	–	–	–	–	–	–	–	–
3	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
5	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–
6	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–
7	–	+	–	+	–	–	–	–	–	–	–	+	–	–	–
8	–	–	–	–[c]	+	–	–	–	–	–	–	–	–	–	–
9	+	+	–	+	–	–[d]	–	+	–	+	–	–	–	–	–
10	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
11	–	–	–	–	–	–	–	–	–	–	–	x[e]	–	–	–
12	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
13	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–
15	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–
FDA method															
1	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
3	+	+	+	+	–	–	–	–	+	+	–	–	–	–	–
5	–[f]	–[f]	+	–[f]	+	–	+	–[f]	–[f]	–	–[f]	–	–[f]	–[f]	–[f]
6	+	–[f]	–[f]	+	+	–[f]	–[f]	–[f]	–[f]	–[f]	–[f]	–[f]	–[f]	–[f]	–[f]
7	+	+	+	+	+	–	+	–	+	+	–	–	–	–	–
8	+	–	–	+	+	–	–	+	+	–	–	–	–	–	–
9	–[f]	–[f]	–[f]	–[f]	–[f]	–[f]	–[f]	–[f]	–[f]	–[f]	–[f]	–[f]	–[f]	–[f]	–[f]
10	–	–[f]	–	–	–	–[f]	–	–	–	–	–	–	–[f]	–	–
11	+	–	+	+	+	–	–	–	–	+	–	x[e]	–	–	–
12	+	+	–[f]	–[f]	–[f]	–[f]	–[f]	–[f]	–[f]	–[f]	–[f]	–[f]	–[f]	–[f]	–[f]
13	–	–	–[f]	–	–	–	–	+	–	–	–	–	+	–	–
15	+	+	+	–	+	–	–	–	–	–	+	–	–	–	–

[a] Most probable number for the following: high inoculum samples, 0.24/g; low inoculum samples, 0.24/g; and control samples, <0.003/g.
[b] +, *Listeria* detected in sample; –, *Listeria* not detected in sample.
[c] The DNAH assay was negative, but *Listeria* was recovered from the assay broth.
[d] The DNAH assay was positive and the assay confirmation was negative.
[e] No sample.
[f] Selective plates had colonies typical of *Listeria*, but were not *Listeria* on confirmation.

Sensitivity and specificity rates for the culture and DNAH methods were calculated according to the McClure method (9). Sensitivity is the number of positives determined by the method divided by the total number of positive samples. Specificity is the number of negatives determined by the method divided by the number of negative samples. The incidence of false negatives among positive samples is 1 minus the sensitivity rate. The incidence of false positive assays among negative tests is 1 minus the specificity rate.

Agreement between the DNA and culture methods was the fraction of the test samples which tested the same by both methods.

993.09 *Listeria* in Dairy Products, Seafoods, and Meats—Colorimetric Deoxyribonucleic Acid Hybridization Method (GENE-TRAK *Listeria* Assay) First Action 1993

Method is test procedure for presence of *Listeria* species in dairy products, meats, and seafoods. Because certain percentage of false positive reactions may be encountered, positive assays must be confirmed by standard culture methods (*see* I).

(*Caution*: *Listeria monocytogenes* infection can cause fetal death. It is recommended that pregnant women avoid handling this organism.)

Table 5. Results of crabmeat analyses for the presence of *Listeria*[a]

	High inoculum samples					Low inoculum samples					Control samples				
Lab.	2	6	9	11	13	1	3	4	7	14	5	2	10	12	15
						DNAH method									
1	+[b]	+	+	–	+	–	–	–	–	–	–	–	–	–	–
2[c]	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–
3	+	+	+	+	+	–	+	+	–	+	–	–	–	–	–
5	+	+	+	+	+	+	–	–	+	–	–	–	–	–	–
6	+	+	+	+	+	+	+	+	–	+	+	–	–	–	–
7	+	+	+	+	+	+	+	–	+	+	–	–	–	–	–
8	–	+	+	–	+	–	–	+	–	–	–	–	–	+	–
10	+	+	+	+	+	+	+	–	+	+	–	–	–	–	–
11	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–
13	+	+	+	+	+	–	–	–	–	+	–	–	–	+	–
16	+	+	+	+	+	+	+	+	–	+	–	–	–	–	–
18	+	+	+	+	+	+	–	–	+	+	–	+	–	–	–
19	+	–	–	+	+	+	+	–	–	+	–	–	–	–	–
						FDA method									
1	+	+	+	+	+	+	+	+	–	+	–	–	+	–	–
2	+	+	+	+	+	+	+	+	–	+	–	–	–	–	–
3[c]	–[d]	–	–	–	–	–	–[d]	–	–	–[d]	–	–	–[d]	–[d]	–[d]
5[c]	–[d]	–	–	–[d]	–	–	–	–	–	–[d]	–	–[d]	–	–[d]	–[d]
6	+	+	+	+	+	–	–	–	+	+	–	–	–	–	–
7	+	+	+	–[d]	+	–[d]	–	–	–	–	–[d]	–	–	–[d]	–[d]
8	+	+	–	+	–	+	+	–	–	–	–	–	–[d]	–	–[d]
10	–	+	–[d]	–	–[d]	–	–	–[d]	–[d]	–	–[d]	–	–	–	–
11	+	+	+	+	+	–	–	–	–	–[d]	–	–	–	–	–
13[c]	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
16	+	+	+	+	+	+	+	+	–	+	–	–	–	–	+
18	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–
19	+	–[d]	+	+	+	+	–	+	–	–	–	–	–	–	–[d]

[a] Most probable number for the following: high inoculum samples, 0.24/g; low inoculum samples, 0.24/g; and control samples, <0.003/g.
[b] +, *Listeria* detected in sample; –, *Listeria* not detected in sample.
[c] Laboratory results were statistical outliers.
[d] Selective plates had colonies typical of *Listeria*, but were not *Listeria* on confirmation.

A. Principle

Detection of *Listeria* ribosomal RNA (rRNA) uses specific DNA probes. Following primary and secondary enrichment of test samples, bacteria are lysed, and labeled *Listeria*-specific DNA probes are added for solution phase hybridization. If *Listeria* rRNA is present in test sample, fluorescein-labeled detector probe and polydeoxyadenylic acid (poly dA)-tailed capture probe will hybridize to target rRNA sequences. Polydeoxythymidylic acid (poly dT)-coated plastic dipstick (solid phase) is then introduced into hybridization solution. Base pairing between poly dA and poly dT facilitates capture of probe:target hybrid nucleic acid molecules onto solid support. Unbound probe is removed by washing, and dipsticks are incubated in horseradish peroxidase–antifluorescein conjugate solution. Conjugate binds to fluorescein label present on hybridized detector probe. Unbound conjugate is washed away, and dipsticks are incubated in substrate–chromogen solution. Reaction of horseradish peroxidase with substrate converts chromogen to blue compound. Reaction is stopped with acid, which changes color of chromogen to yellow. Absorbance at 450 nm is measured. Absorbance in excess of threshold value indicates presence of *Listeria* in test samples.

B. Method Performance

See table of method performance data, **993.09**.

C. Apparatus

(**a**) *Photometer.*—Capable of measuring absorbance at 450 nm of 1 mL solution in 12 × 75 mm tubes in reference and sample wells.

(**b**) *Tube racks.*—3 plastic, heat-resistant (to 65°) racks, to hold ≥50 tubes (12 × 75 mm). Minimum of 5 wells per row with 18 mm spacing between wells (measured between centers of wells).

Table 6. Results of frankfurter analyses for the presence of *Listeria*[a]

	High inoculum samples					Low inoculum samples					Control samples				
Lab.	2	6	9	11	13	1	3	4	7	14	5	8	10	12	15
							DNAH method								
1	–[b]	+	+	–	+	–	+	+	–	–	–	–	–	–	–
2	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–
3	+	+	–	+	+	–	+	+	+	+	–	–	–	–	–
5	+	+	+	+	+	+	–	–[c]	+	+	–[c]	–	–	–	–
6	+	+	+	+	+	+	–	+	+	+	–	–	–	–	–
8	–	+	+	+	–	+	+	+	–	+	–	–	–	–	–
9[d]	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
10	+	+	–[c]	–	+	+	+	+	–[c]	–[c]	–	–[c]	–[c]	–[c]	–[c]
11[d]	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
13[d]	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–
14	+	–	–	–	–	+	+	–	–	–	–	–	–	–	–
15	+	+	+	+	+	+	+	–	+	+	–	–	–	–	–
17	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–
							USDA method								
1	+	+	+	+	+	–	+	+	+	+	–	–	–	–	–
2	+	–	+	+	+	–	+	+	+	+	–	–	–	–	–
3	+	+	+	+	+	–	+	–	+	+	–	–	–	–	–
5	+	+	+	–	+	+	+	+	+	–	–	–	–	–	–
6	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–
8	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–
9	+	+	–	+	+	+	+	+	+	+	–	–	–	–[e]	–[e]
10	+	+	+	–	+	+	+	–	+	–	–	–	–	–	–
11	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–
13	+	–[e]	+	+	+	+	–[e]	+	+	+	–	–	–	–	–
14	+	–[e]	–[e]	+	–	+	+	–[e]	–	+	–	–	–	–	–
15	+	+	+	+	+	+	–	–	+	+	–	–	–	–	–
17	+	+	+	+	+	+	+	+	+	+	–	–	–[e]	–[e]	–[e]

[a] All frankfurter samples were naturally contaminated. Most probable number for the following: high level samples, 0.043/g; low level samples, 0.015/g; and control samples, <0.003/g.
[b] +, *Listeria* detected in sample; –, *Listeria* not detected in sample.
[c] The DNAH assay was positive and the assay confirmation was negative.
[d] Laboratory results were statistical outliers.
[e] Selective plates had colonies typical of *Listeria*, but were not *Listeria* on confirmation.

(**c**) *Dipstick holders.*—Plastic device to hold 5 dipsticks in row with 18 mm spacing between dipsticks (center to center).

(**d**) *Wash basins.*—4 metal, or plastic, heat-resistant (to 65°) basins, 10 × 10 × 9 cm, with covers.

(**e**) *Tubes.*—Glass, 12 × 75 mm.

(**f**) *Water baths.*—*(1)* Capable of maintaining 65 ± 1°, to hold 1 tube rack, 1 wash basin, with 5 cm water level. *(2)* Capable of maintaining 37 ± 1°, to hold 1 tube rack with 5 cm water level.

(**g**) *Repeater pipet.*—Capable of accurately delivering 0.1, 0.25, and 0.75 mL, with syringe-barrel tips (optional). Alternatively, serological pipets may be used.

(**h**) *Sterile capped tubes.*—To contain 1 mL volume.

(**i**) *Sterile cotton applicator swabs.*

Items (**a**)–(**d**) are available from GENE-TRAK Systems (31 New York Ave., Framingham, MA 01701). Substitute materials from other sources must be tested for equivalence.

D. Reagents

(*Caution*: Probe solution and positive control solution contain 0.1% sodium azide. Disposal of this reagent into sinks with copper or lead plumbing should be followed immediately with large quantities of water to prevent potential explosion hazards.)

Store pretreatment reagent concentrate, lysis reagent concentrate, probe solution, enzyme conjugate 100× concentrate, substrate solution, chromogen solution, positive control solu-

Table 7. Results of roast beef analyses for the presence of *Listeria*[a]

	High inoculum samples					Medium inoculum samples					Low inoculum samples				
Lab.	1	5	6	9	12	2	7	8	13	14	3	4	10	11	15
							DNA method								
1	+[b]	+	+	+	+	+	–	+	+	+	–	+	+	+	–
2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
3	+	+	+	+	+	+	+	+	–	+	+	+	+	+	+
4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–
5	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+
6	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
8	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
9	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
10	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+
11	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–[c]
13	+	+	+	+	+	+	+	+	+	+	+	–	–	+	+
14	+	+	+	+	+	+	+	+	–[c]	+	+	+	+	–	–
15	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
17	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
							USDA method								
1	+	+	+	+	+	+	+	+	+	+	–	+	+	+	+
2	+	+	+	+	+	+	+	+	+	+	–	+	+	+	+
3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
4	+	+	+	+	+	+	+	+	+	–	+	+	+	+	+
5	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+
6	+	+	+	+	+	+	+	+	–	+	+	+	+	–[d]	–[d]
7	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
8	+	+	+	+	+	+	+	+	+	+	–	+	+	+	+
9	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
10	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
11	+	+	–[d]	+	+	+	+	+	–	+	–[d]	+	+	+	+
13	+	+	+	+	+	+	+	+	+	+	+	–	–	+	+
14	+	+	+	+	+	+	–[d]	+	–[d]	–	+	–	–	–	–
15	+	+	+	+	+	+	+	+	–	+	–	–	+	+	+
17	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–

[a] All roast beef samples were naturally contaminated. Most probable number for the following: high inoculum samples, 0.46/g; medium inoculum samples, 0.093/g; and low level samples, 0.023/g.
[b] +, *Listeria* detected in sample; –, *Listeria* not detected in sample.
[c] The DNAH assay was positive and the assay confirmation was negative.
[d] Selective plates had colonies typical of *Listeria*, but were not *Listeria* on confirmation.

tion, and negative control solution at 2–8°. Store all other solutions and dipsticks at room temperature (<30°).

(a) *Pretreatment reagent concentrate.*—150 mg Lysozyme and 3000 units mutanolysin in 0.1M potassium phosphate buffer.

(b) *Pretreatment reagent buffer.*—0.1M Tris pH 7.1–7.4, 0.01M disodium ethylenediamine tetraacetate (EDTA), and 0.0075% bromphenol blue.

(c) *Lysis reagent concentrate.*—Serine protease derived from *Tritirachium album* (Proteinase K is suitable).

(d) *Lysis reagent buffer.*—5% *n*-lauroyl sarcosine (Sarkosyl is suitable), 0.005M EDTA, 0.26M Tris pH 7.2–7.6, 1.0M NaCl, and 0.05% brilliant yellow.

(e) *Listeria probe solution.*—Fluorescein-labeled, *Listeria*-specific, synthetic oligonucleotide DNA probe and polydeoxyadenylic acid (dA)-tailed, *Listeria*-specific, synthetic oligonucleotide DNA probe in 0.1M Tris, pH 7.5; 0.001M EDTA; 0.1% bovine serum albumin; 0.01% octyl phenol ethylene condensate, nonionic detergent (NP-40 is suitable); 0.2% cresol red; and 0.1% sodium azide. Probes must exhibit specificity for *Listeria* and lack of cross-reactivity with bacteria of other genera. Specificity is determined by testing pure cultures of selected bacteria, grown in non-selective media to titer $\geq 10^7$/mL, in assay. Test panel for specificity should include multiple strains of all *Listeria* species and strains of other bacteria which may be present in dairy products, seafoods, and meats.

Table 8. Results of ground pork analyses for the presence of *Listeria*[a]

	High inoculum samples					Medium inoculum samples					Low inoculum samples				
Lab.	5	6	11	12	15	1	2	7	8	13	3	4	9	10	14
						DNAH method									
1	+[b]	+	+	+	+	+	+	+	+	+	+	+	+	–	+
3	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–
5	+	+	+	+	+	+	+	+	–	+	+	+	+	+	+
6	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+
8	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
9	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+
10	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
11[d]	+	+	+	+	+	+	+	–[c]	+	+	–[c]	–[c]	–[c]	–[c]	+
13	+	+	+	+	+	+	+	+	+	+	–	+	+	–[c]	+
14	+	+	+	+	+	+	+	+	+	+	+	–	+	–[c]	+
15	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
17	+	+	+	+	+	+	+	+	+	+	+	+	–[c]	–	+
						USDA method									
1[d]	+	+	–	–	–	+	+	–	+	–	+	+	+	–	–[e]
3	+	–[e]	+	+	+	+	+	+	–[e]	+	+	–[e]	+	+	–[e]
5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
6	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7	+	+	+	+	+	+	+	–	+	+	+	+	+	–	+
8	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
9	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
10	+	–	–	+	+	–	+	+	+	+	+	+	+	–	+
11	–[e]	+	+	+	+	+	+	–[e]	+	+	+	–[e]	+	–[e]	+
13	+	+	+	+	+	+	+	+	+	+	+	+	–[e]	+	+
14	+	+	+	+	+	–	+	+	+	–	–[e]	+	+	+	+
15	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
17	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+

[a] All ground pork samples were naturally contaminated. Most probable number for the following: high level samples, 0.24/g; medium level samples, 0.24/g; and low level samples, 0.024/g.
[b] +, *Listeria* detected in sample; –, *Listeria* not detected in sample.
[c] The DNAH assay was positive and the assay confirmation was negative.
[d] Laboratory results were statistical outliers.
[e] Selective plates had colonies typical of *Listeria*, but were not *Listeria* on confirmation.

(**f**) *Wash solution, 20× concentrate.*—1.0M Tris, pH 7.5; 0.4M EDTA; 3.0M NaCl; and 0.2% Tween-20.

(**g**) *Enzyme conjugate, 100× concentrate.*—Horseradish peroxidase–antifluorescein polyclonal antibody conjugate.

(**h**) *Substrate solution.*—Urea peroxide.

(**i**) *Chromogen solution.*—Tetramethylbenzidine.

(**j**) *Stop solution.*—2.0M H_2SO_4. (*Caution*: Corrosive. Avoid contact with skin; if contact occurs, wash skin thoroughly with water.)

(**k**) *Dipsticks.*—Polystyrene dipsticks, 8 cm (5 cm handle, 3 cm fin). Fin has 5 paddle-like protrusions coated with polydeoxythymidylic acid (dT). Binding capacity of dT-coated dipsticks should exceed 250 ng of complementary sequence, (dA). Dipsticks should be tested in combination with matrix of other reagents to ensure proper method sensitivity.

(**l**) *Positive control solution.*—*Listeria*-specific oligonucleotide DNA at 20 ng/mL total concentration (sufficient to produce absorbance value ≥1.0 when tested in assay) in 0.1M Tris, pH 7.5; 0.001M EDTA; 0.1% bovine serum albumin; 0.01% nonionic detergent (NP-40); and 0.1% sodium azide.

(**m**) *Negative control solution.*—Formaldehyde-inactivated *Streptococcus faecium* in phosphate-buffered saline, (**n**)*(2)*, in concentrations sufficient to produce absorbance value >0.15 in assay when stringency conditions of assay (hybridization and/or wash temperatures) are not correct. Correctly performed assay should yield absorbance value ≤0.15 for negative control. Actual cell concentration used may vary depending on strain of organism employed and media and conditions used for its preparation. Also contains 0.05% 2-bromo-2-nitropropane-1,3-diol (Bronopol).

Table 9. Results for DNAH method and FDA/USDA culture method for detection of *Listeria* in inoculated foods

		Results in agreement		False negative results		Unconfirmed positive assays	
Food	Level	Positive	Negative	DNAH	Culture	DNAH	Culture
2% Milk	high	51	1	10	3	0	4
	low	46	4	10	5	1	1
	control	0	64	0	0	1	0
Brie cheese	high	57	1	1	1	1	2
	low	51	1	2	6	3	3
	control	0	60	0	0	1	4
Crab	high	33	1	9	22	0	7
	low	8	23	12	22	0	7
	control	0	59	2	4	0	13
Frankfurters	high	39	6	18	2	1	3
	low	30	7	23	5	3	2
	control	0	65	0	0	5	5
Roast beef	high	74	0	0	1	0	1
	medium	66	1	2	6	1	2
	low	55	6	4	10	1	3
Ground pork	high	58	0	0	7	0	2
	medium	56	1	1	7	1	2
	low	44	6	10	5	7	7

(**n**) *Phosphate-buffered saline (PBS) solutions.—(1) 10× stock solution.*—Dissolve 12.0 g Na_2HPO_4, 2.2 g NaH_2PO_4, and 85.0 g NaCl in H_2O to 1 L. Autoclave 15 min at 121°. *(2) Working solution.*—Dilute stock solution 1:9 with sterile H_2O. Mix well. Adjust pH to 7.5 with 0.1N HCl or 0.1N NaOH, if necessary.

(**o**) *Phosphate-buffered Listeria enrichment broth (PEB).*—Combine 30.0 g trypticase soy broth powder, 6.0 g yeast extract, 1.35 g KH_2PO_4, 9.6 g Na_2HPO_4, and 1 L H_2O and autoclave 15 min at 121°. Add 15 mg acriflavine HCl, 40 mg nalidixic acid, and 50 mg cycloheximide to sterile media just before use. Stock solutions, 0.5% (w/v), of acriflavine HCl and nalidixic acid are prepared in H_2O and filter-sterilized. Stock solution, 1% (w/v), of cycloheximide is prepared in 40% EtOH and filter-sterilized.

(**p**) *UVM-2 broth.*—Dissolve 5.0 g proteose peptone, 5.0 g tryptone, 5.0 g lab Lemco powder, 5.0 g yeast extract, 20.0 g NaCl, 1.35 g KH_2PO_4, 12.0 g Na_2HPO_4, 1.0 g esculin, and 1.0 mL nalidixic acid solution (2% in 0.1N NaOH) in 1 L H_2O. Autoclave 15 min at 121°. Do not overheat. Store in refrigerator. Just before use, add 1.0 mL of 2.5% filter-sterilized acriflavine per L medium.

(**q**) *Modified UVM-2 broth.*—Add 5.0 g/L LiCl to UVM-2 broth before autoclaving [*see* (**p**)].

(**r**) *Modified LCA agar.*—Combine 52 g brain heart infusion agar, 10 g LiCl, 10.0 g glycine anhydride, and 1 L H_2O and autoclave 15 min at 121°. Cool to 45–50° and add 5.0 mL filter-sterilized ceftazidime solution (10 mg/mL). Stir molten agar with magnetic mixing bar and pour 20 mL portions into 100 mm diam. Petri dishes. Store LCA agar plates refrigerated in plastic bags. Plates may be stored 1 month at 4°. Prepare 10 mg/mL ceftazidime solution by dissolving 50 mg ceftazidime in 5.0 mL H_2O. Discard any remaining ceftazidime solution.

(**s**) *Diagnostic reagents.*—Necessary for culture confirmation of positive DNA hybridization assays [refer to current edition of Bacteriological Analytical Manual (BAM), Arlington, VA].

Items (**a**)–(**m**) are available as Colorimetric GENE-TRAK™ *Listeria* Assay (DNA Hybridization Test for Detection of *Listeria*) from GENE-TRAK Systems.

E. General Instructions

Include 1 positive control and 1 negative control with each group of test samples.

Do not touch fin portion of dipstick with fingers; hold by handle only. Do not reuse dipsticks or wash solution.

Use separate pipets or tips for each sample and kit reagent to avoid cross-contamination. Exercise care not to contaminate substrate–chromogen mixture with enzyme conjugate.

Return reagents requiring refrigeration to 2–8° immediately after use. Refer to storage requirements on individual reagent bottle labels.

Treat all materials in contact with bacterial cultures as biohazardous material and decontaminate by appropriate methods.

F. Sample Preparation

(1) Primary enrichment.—Proceed according to product type as follows:

Dairy products.—For solid and semisolid products, aseptically weigh 25 g sample into sterile high-speed blender jar. Add 225 mL sterile PEB, **D(o)**, prewarmed to 35°, and blend 2 min at 10 000–12 000 rpm. For liquid samples, add 225 mL sterile PEB, prewarmed to 35°, and shake gently. Incubate 24 ± 4 h at 35°.

Red meats and poultry.—Aseptically weigh 25 g sample into sterile high-speed blender jar. Add 225 mL sterile UVM-2 broth, **D(p)**, prewarmed to 35°, and blend 2 min at 10 000–12 000 rpm. Incubate 24 ± 4 h at 35°.

Seafoods.—Aseptically weigh 25 g sample into sterile high-speed blender jar. Add 225 mL sterile Modified UVM-2 broth, **D(q)**, prewarmed to 35°, and blend 2 min at 10 000–12 000 rpm. Incubate 24 ± 4 h at 35°.

(2) Secondary enrichment (all sample types).—Mix incubated primary enrichment culture well and dip sterile cotton swab into culture. Swab onto entire surface of LCA agar plate, expressing as much liquid from swab as possible. Incubate LCA plate 24 ± 2 h at 35°. Harvest growth from LCA plate with sterile swab and resuspend by swirling swab vigorously 5 s in 1 mL PBS, **D(n)**, in sterile, capped tube. Express as much liquid from swab as possible before discarding swab.

G. DNA Hybridization Assay

(1) Fill water baths ca 5 cm and adjust to 37 ± 1° and 65 ± 1°.

(2) Prepare pretreatment reagent by adding 12 mL pretreatment reagent buffer, **D(b)**, to pretreatment reagent concentrate, **D(a)**, and mix by gentle swirling. Place on ice.

(3) Prepare lysis reagent by adding 6 mL lysis reagent buffer, **D(d)**, to lysis reagent concentrate, **D(c)**, and mix by gentle swirling. Place on ice.

(*Note*: Reconstituted pretreatment reagent and lysis reagent are stable 60 days stored at –20°. To thaw, place bottles in 37° water bath 10 min, then place on ice.)

(4) For each 25 tests performed: dilute 65 mL 20× wash solution concentrate, **D(f)**, to 1235 mL H_2O; prepare 1 wash basin with 300 mL 1× wash solution, cover, and place in 65° water bath; and prepare 3 wash basins with 300 mL 1× wash solution at room temperature and cover basins until needed.

(5) Label 12 × 75 mm tubes for samples, plus 1 positive control, and 1 negative control. Place tubes in rack in rows of 5.

(6) Vortex or otherwise mix each PBS growth resuspension, **F***(2)*, positive control solution, **D(l)**, and negative control solution, **D(m)**. Add 0.5 mL to tubes as labeled. Return controls to 2–8°.

(7) Add 0.10 mL reconstituted pretreatment reagent, *(2)*, to each tube. Shake rack of tubes by hand 5 s. Incubate tubes 15 min in 37° water bath. (*Note*: If, after addition of pretreatment reagent, resulting solutions are not purple, recheck that pretreatment reagent has been added.)

(8) Without removing rack from water bath, add 0.10 mL reconstituted lysis reagent, *(3)*, to each tube. Briefly remove rack from water bath and shake rack by hand 5 s. Incubate tubes 15 min in 37° water bath. (*Note*: If, after addition of lysis reagent, resulting solutions are not green, recheck that lysis reagent has been added.)

(9) Place 1 dipstick for each tube into dipstick holders. Rinse dipsticks 2–3 min in 1× wash solution at room temperature. Remove excess solution by blotting to absorbent paper (touch tip of dipstick fin to paper).

(10) Add 0.10 mL probe solution, **D(e)**, to each tube. Place dipsticks into sample tubes. Mix contents in tubes by raising and lowering dipsticks 5×.

(*Note*: If, after addition of probe solution, resulting solutions are not red, recheck that probe solution has been added.)

(11) Move rack of tubes to 65° water bath and incubate 1 h.

(12) Set up and label second rack of 12 × 75 mm tubes. Prepare sufficient 1× enzyme conjugate by mixing 100× enzyme conjugate concentrate, **D(g)**, and 1× wash solution 1:100. Dispense 0.75 mL 1× enzyme conjugate into each empty tube.

(13) Remove dipsticks from tubes in 65° water bath. Wash dipsticks sequentially, with gentle shaking 1 min each, first in 65° wash solution (do not remove wash basin from 65° water bath), then in room temperature wash solution. Blot dipsticks on absorbent paper. Place dipsticks into second set of tubes containing enzyme conjugate. Incubate 20 min at room temperature.

(14) Set up and label third rack of 12 × 75 mm tubes. Add 1 tube for blank. Prepare substrate–chromogen mixture consisting of 2 parts substrate solution, **D(h)**, to 1 part chromogen solution, **D(i)**. Dispense 0.75 mL substrate–chromogen mixture into each empty tube.

(15) Remove dipsticks from enzyme conjugate tubes. Wash dipsticks sequentially with gentle shaking 1 min each in remaining 2 basins containing room temperature 1× wash solution. Blot dipsticks on absorbent paper. Place dipsticks into tubes containing substrate–chromogen mixture. Incubate 30 min at room temperature. Remove dipsticks from tubes and discard.

(16) Add 0.25 mL stop solution, **D(j)**, to each tube containing substrate–chromogen mixture, including blank. Shake rack by hand to mix tube contents.

(17) To measure absorbance value, *A*, (at 450 nm), wait for reading to stabilize before recording result for each tube. Determine negative control absorbance by placing tube labeled "Blank" in reference well and negative control tube in sample well. Determine positive control absorbance by placing tube labeled "Blank" in reference well and positive control tube in sample well. Determine test sample absorbance by placing negative control tube in reference well and test sample tube in sample well.

H. Data Analysis

A for negative control should be ≤0.15; *A* for positive control should be ≥1.00. If these results are not obtained, assay should be repeated.

Negative criterion.—Test sample is considered negative (nonreactive for presence of *Listeria*) if *A* is ≤0.10.

Positive criterion.—Test sample is considered positive (reactive for presence of *Listeria*) if *A* is >0.10.

I. Confirmation of Positive DNA Hybridization Results

Samples found positive by DNA hybridization assay must be confirmed by standard culture methods. For confirmation, streak PBS growth resuspension on a *Listeria* selective plating medium and continue with biochemical identification of pre-

sumptive *Listeria* isolates according to standard methods in current edition of BAM.

Ref.: JAOAC 77, May/June (1994)

Results and Discussion

Nineteen laboratories participated in the study (Table 1). Each laboratory tested 1 or more food types. Delays related to shipping were experienced by collaborators 4 and 12 for 2 food types. Since the samples were not received in time to begin the analysis on the specified date, the samples were not analyzed. Some laboratories were unable to complete the sample analyses even though samples were received in time. Laboratory 2 began the analysis of the pork samples at a later time and submitted results in agreement with those submitted by other laboratories. These data are not shown or included in the method performance statistics.

Some laboratory data sets were identified as outliers according to the procedure of McClure (9). These data (Tables 2-9), were outliers because the reporting laboratory, compared to the other laboratories, detected *Listeria* in proportionally fewer samples. The typical outlier did not detect *Listeria* in any or very few samples. This observation does not appear to be laboratory, food, or method related and has been observed in another collaborative study of a *Listeria* method by one of the authors (Curiale) and in a pilot study (10). Failure to detect *Listeria* occasionally appears to be an integral part of current methodology. Excluding outlier data from statistical calculations overestimates efficacy of the methods. Consequently, these data have not been excluded although they have been noted.

Milk

Test results for analyses of the milk samples were received from 13 laboratories (Table 1). Sample 6 was missing from the set sent to Laboratory 7, hence no results for this sample were available. The results from Collaborators 6 and 15 were determined to be outliers for the DNAH method (Table 2). Both laboratories reported significantly fewer DNAH positive samples compared to the other laboratories.

Of the 65 test samples prepared with a high inoculum of *Listeria*, 54 were positive by the DNAH method and 61 were positive by the FDA culture method. *Listeria* was recovered from the assay broths of 2 DNAH method negative samples. Since DNAH method negative samples contained *Listeria*, the levels of the organism in 2 negative suspensions were probably below the detection limit of the assay. Since the corresponding FDA method results for these samples were positive, the DNAH method results are counted as false negatives. For the analysis by the culture method, the laboratories reporting the 4 negative samples found typical colonies on the isolation plates. These isolates were not *Listeria* according to the biochemical confirmation tests. The failure to confirm a positive assay, either the plate reading assay of the culture method or the DNA hybridization assay of the DNAH method, constitutes a false positive result. For samples containing the low inoculum of *Listeria*, 51 of 65 were positive according to the DNAH method and 56 of 65 were positive by the FDA culture method. One DNAH method positive did not confirm by culturing and 1 DNAH method negative sample confirmed the presence of *Listeria*. One FDA sample exhibited typical colonies on the isolation media, but they were not identified as *Listeria*.

There were 64 control samples of milk without added *Listeria*. All were negative by both the DNAH and FDA culture methods. There was 1 positive DNAH assay among these samples that did not confirm upon culture analysis.

All of the laboratories correctly identified *L. innocua* as the strain used for inoculation. In addition, laboratory 1 reported 1 or more isolations of *L. monocytogenes* and laboratory 13 reported finding *L. welshimeri*.

Brie Cheese

Two sets of Brie cheese samples were sent to collaborators. The number of positives in the first set was very low: there were 3 DNAH and 1 culture method positives among the 50 high inoculum samples and 1 DNAH and 0 culture method positives among the 50 low inoculum samples (data not shown). The number of positives in this set was too low for evaluation of the methods. The second set of samples was prepared and sent to 13 collaborators (Table 1). Collaborator 10 was unable to complete the analysis. Thus, 12 valid data sets were available for comparison of the methods (Table 3). The data from laboratory 14 for the DNAH method was a statistical outlier.

Among the samples containing the high inoculum of *Listeria*, 59 of 60 samples were positive by either the DNAH method or the FDA culture method. At the lower inoculum level, 57 of 60 were positive by DNAH and 53 of 60 were positive by the culture method. All 60 control samples were negative for *Listeria* according to both methods. [Of the negative samples, 1 high, 3 low and 1 control were positive by DNAH assay (not considering confirmation) and 2 high, 3 low, and 4 control samples had colonies typical for *Listeria* by the FDA culture method.]

All but 1 of the collaborators correctly identified *L. monocytogenes* in the inoculated samples. Laboratory 14 reported the presence of *L. innocua* in addition to *L. monocytogenes*.

Crabmeat

Two sets of crabmeat samples were prepared. The first set was sent to 13 collaborators. The second set was sent to 14 laboratories after the enrichment protocol for seafood was modified.

From the first shipment 12 valid data sets were received (Table 4). Eight of 60 high inoculum level *Listeria* samples were positive according to the DNAH method and 28 of 60 were positive by the FDA culture method. One sample was negative by the DNAH method and positive for *Listeria* by isolation of the organism from the LCA plate suspension. At the lower *Listeria* level, 3 of 60 were positive by the DNAH method and 10 of 60 were positive by culture method. For all 3 inoculation levels, about 50% of the culture method negative samples exhibited typical colonies on the isolation plates which were not identified as *Listeria*. In contrast, only 1 DNAH assay positive sample was not identified as containing *Listeria*.

The large difference in the number of positive samples between the DNAH and FDA culture methods for the crabmeat sample set in this study was not anticipated, since the methods were comparable in a pilot study (5). Attempts to reproduce the poor results of the DNAH method for the first crabmeat set in this study were unsuccessful (Bottari et al., communication). Thus, it was concluded that the results for the crab were most probably lot specific. While attempting to reproduce the results, a modified UVM-2 enrichment broth for the DNA method was also evaluated. The modified DNAH method was as productive as the FDA method and the original DNAH method with unmodified UVM-2 in a repeat of the pilot study for seafoods (Bottari et al., communication). The modified UVM-2 broth enrichment procedure was adopted for the DNAH method because the additional 5 g LiCl per 1 L makes the medium more selective.

The second set of crabmeat samples for use with the modified enrichment for the DNAH method was shipped to 14 laboratories (Table 1) and 13 of them reported results. The DNAH method results from 1 laboratory and the FDA culture results from 3 other laboratories were statistical outliers (Table 5). The DNAH method detected *Listeria* in 55 of 65 high inoculum samples and 30 of 65 low inoculum samples, and 4 of 65 control samples. By the FDA culture method, 42 of 65 high inoculum, 20 of 65 low inoculum, and 2 of 65 control samples were positive for *Listeria*. *L. monocytogenes* was found in all positive samples.

Frankfurters

Frankfurter sample sets were sent to 13 laboratories (Table 1). Three laboratories reported significantly fewer positive samples by the DNAH method (Table 6). These data sets were outliers according to the statistical test.

Of the 65 test samples from the high inoculum batch, 41 samples were positive for *Listeria* by the DNAH method and 57 were positive by USDA culture. For the low inoculum samples, 35 were DNAH positive and 53 were culture positive. All positive samples contained *L. monocytogenes*. The 65 control samples tested negative for *Listeria* by both the DNAH and culture procedures.

DNAH assays that were positive but failed to confirm the presence of *Listeria* by isolation were recorded for 1 high inoculum, 3 low inoculum and 5 control samples. For the USDA plate assay there were 3 high, 2 low, and 5 control samples that produced suspect colonies but did not confirm as *Listeria* upon further analysis.

Roast Beef

Samples of roast beef were sent to 15 laboratories (Table 7). *Listeria* in the low inoculum sample was a natural contaminant. The medium and high inocula samples contained the natural contaminant plus the *Listeria* strain used for inoculation.

All 75 high inoculum samples were positive by DNAH and 74 of 75 samples were positive by the USDA culture method. Both methods isolated *L. monocytogenes* as the contaminating organism; only 1 isolation of *L. innocua* was reported.

For the medium inoculum *Listeria* samples, 72 of 75 were positive by the DNAH method and 68 of 75 were positive according to the culture method. *L. monocytogenes* was the most commonly found *Listeria* isolated from these samples, although single isolations of *L. innocua* and *L. seeligeri* and 2 isolations of *L. murrayi* were reported.

For the low inoculum samples naturally contaminated with *Listeria*, 65 of a possible 75 were positive by DNAH and 59 of 75 were positive by culture analysis. *L. monocytogenes* was the most commonly isolated *Listeria* species; *L. innocua* was isolated 5 times and *L. seeligeri* was isolated once.

Raw Ground Pork

Natural contamination of raw ground pork by high level of *Listeria* is common. In addition, these samples contain high levels of natural flora. This features make pork a useful test matrix for the evaluation of a method's sensitivity, even though raw meat is not normally analyzed for *Listeria*.

Samples were sent to 16 laboratories (Table 1). Thirteen data sets were available for analysis. The results from laboratory 11 for the DNAH method and the results from laboratory 1 for the USDA method were statistical outliers (Table 8).

All 65 naturally contaminated samples containing high level *Listeria* were identified as positive using DNAH (Table 8), and 58 using the culture method. At the medium contamination level, 63 samples of 65 were identified as positive by DNAH and 57 by the culture method. Upon analysis of the low level naturally contaminated samples, 49 of 65 were positive by the DNAH procedure and 54 by USDA culture.

The most common isolate of *Listeria* in the high contamination level samples was *L. innocua* which accounted for 88% of the identifications. *L. monocytogenes* and *L. grayi* were the other isolates found. About 35% of the *Listeria* isolated from the medium contamination level samples was *L. innocua* and 48% was *L. monocytogenes*. Also found were: *L. welshimeri*, *L. murrayi*, and *L. grayi*. *L. innocua* accounted for 65% of the isolates in the low contamination level samples and *L. monocytogenes* for another 25%. The remaining isolates were either *L. welshimeri* or *L. grayi*. All *L. grayi* identifications were from collaborator 13, and it was the most commonly identified *Listeria* from this laboratory for all pork samples.

Method Performance

The results from Tables 2–8 are summarized in Table 9. Table 9 values were used to calculate the method performance and are presented in Table 993.09. Statistical methods for the comparison of 2 or more collaborative studied methods are not available. Thus, the evaluation of comparative performance are by insightful inspections of similarities and trends. Differences of a few percentage points are probably not meaningful. Larger differences may not always be meaningful either. Common problems associated with the analysis of foods are low numbers of organisms and heterogenous distribution of the organisms in the food. Collaborative studies are purposefully designed to test for low numbers. Since the methods examine different portions of the foods, some differences can be ex-

pected. While considerable effort is made to achieve a good distribution of the organisms in the food, it is not always realized and the success is not easily determined. One test portion might contain *Listeria* and another might not. These situations confound simple interpretations of the performance statistics.

For some inoculum levels, the performance statistics suggest that one method performed considerably better than the other. In many instances, this may reflect low levels of organism or unequal distribution. In other, it is exaggerated by the low number of samples used to calculate the performance statistic, rather than by a clear difference in performance. For example, the sensitivity of the DNAH method appears to be 2 times better than the culture method for detection of *Listeria* in the control samples of crabmeat (Table 993.09), but the result is based on only 4 positive samples (Table 9, false-negative columns). A greater number of skewed results from low numbers of samples is seen for the specificity determinations (Table 993.09).

Trends showing that one method performed better than the other on a particular food appear in several places. This is commonly seen in the specificity results, but is likely attributable to the low sample numbers used to calculate the rates. In measurements of sensitivity, the DNAH method using the modified UVM-2 enrichment protocol appeared consistently better for the analyses of the crabmeat, whereas the culture method appeared better for the frankfurters. These trends are not supported by the pilot study data, so they may represent results peculiar to the food lot tested. In the pilot study (5), some discrepancies of equal magnitude were observed. However, other foods were involved and the results favored the DNAH method. These findings suggest the possibility that one or the other method may be superior for a particular food sample lot. However, no data collected to date suggest that either DNAH or culture methods would perform consistently better for a specific food type.

For certain foods, several collaborators reported all samples negative by one method but not by the other method in contrast to results obtained in other laboratories. In a few instances, both methods failed in some laboratories and not others (review outliers in Tables 2, 5 and 6). These results suggest a localized failure of one or the other methods. No obvious failure pattern could be discerned, nor were there any laboratory errors evident. These findings may represent an enrichment failure caused by improper antibiotic or inhibitor concentration.

An advantage of the DNAH method is that negative results are available in 48 h, while the culture method requires 72–96 h. These times are achieved only when the DNAH assay is negative or no suspicious colonies are observed on the culture isolation plates. Longer times are required to confirm positive results. Thus, high numbers of positive assays which do not confirm are undesirable. The incidence of false-positive DNAH assays among total negative tests was low (Table 993.09). Overall, 6.3% of the negative samples required more than 48 h to obtain a negative result. In comparison, 16.5% of the culture negative samples required effort beyond reading the isolation plates. Separating the results for the USDA procedure from the results for the FDA procedure, 21.6% of the negative meat samples required confirmation, as did 14.3% of the dairy, and 15.1% of the crabmeat samples. In the first set of crabmeat samples 44.5% of the negative samples required confirmation (from Table 4). The DNAH method with 13.9% of the negative meat samples requiring confirmation was comparable to the USDA procedure. However, for the *Listeria* negative dairy and seafood items, the DNAH method was inaccurate for 6.6% of the dairy and <1% of the crabmeat samples. For sample sets containing a high proportion of negative samples, these data show that the average test time for the DNAH method was close to the minimum test time of 48 h. In contrast, for culture procedures, the minimum test time frequently exceeded 72 h.

Recommendation

On the basis of the results of this study, it is recommended that the *Listeria* deoxyribonucleic acid hybridization method be adopted first action.

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FOOD BIOLOGICAL CONTAMINANTS

Detection of Pork in Heated Meat Products by the Polymerase Chain Reaction

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A new method for the specific, sensitive, and semiquantitative detection of pork (*Sus scrofa*) in heat-treated meat products has been developed. DNA was isolated from meat samples by using a DNA-binding resin and subjected to polymerase chain reaction (PCR) analysis. First, oligonucleotides yielding a specific 137-base-pair (bp) fragment from eucaryotic DNA amplified from a highly conserved region of the 18-S ribosomal gene was used to assess DNA quality. Second, the presence of pork DNA was determined with specific oligonucleotides yielding a 108-bp fragment amplified from the porcine growth hormone gene. The test detected pork in fresh or heated meat mixtures of pork in beef at levels below 2%. This approach was superior to commercially available immunological tests that were not able to detect levels of pork less than 20% in cooked meat or less than 10% in fresh meat.

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Species differentiation of heated meat products to detect adulteration or fraudulent substitution has been a problem for food laboratories. Consumers want to be protected from falsely labeled meat products that contain unknown, exotic, less desirable or objectionable meat species for economic, religious, and health reasons. Methods currently available for species differentiation include isoelectric focusing in polyacrylamide gels (1), enzyme-linked immunosorbent assays (ELISA) (2), and agar gel immunodiffusion assays (3). Serological tests are specific and sensitive, but cross-reactions of closely related species cannot be excluded. ELISAs based on antibodies to heat-resistant antigens can be used for the confirmation of species in heated meat samples. However, electrophoretic and immunological assays often fail in species detection of heated meat mixtures and other food products with complex matrices.

These problems can be solved with methods based on the presence of species-specific DNA sequences in meat detected by techniques such as DNA hybridization (4–6) and the polymerase chain reaction (PCR) (7). DNA hybridization experiments to detect pig, sheep, goat, and cattle meat components were carried out with ^{32}P-labeled probes made from genomic DNA and could be used for quantitative determinations in the quality control of heat-treated meat products (8). Cross-reactions of closely related species occur with this technique (9) and, even when nonradioactive detection systems are used, become costly and time consuming.

Rapid amplification of specific DNA sequences by PCR should circumvent these problems (10, 11). In this study, we used sequence data for the growth hormone gene of pigs (*Sus scrofa*), cattle (*Bos taurus*), sheep (*Ovis ovis*), and goats (*Capra hircus*) to design porcine-specific oligonucleotides for PCR. The content of pork in prepared mixtures of known composition of raw as well as heated pork and beef was determined. In addition, various commercially available meat products were analyzed. In these experiments, a comparison was made of PCR and DNA hybridization with the ELISA and Ouchterlony double immunodiffusion methods.

Experimental

Meat and Meat Product Samples

Meat samples from various animal species authenticated at slaughter by a veterinarian, heat-treated meat mixtures (pork–beef), and meat products were obtained from the Federation of Migros Cooperatives' Meat Laboratory, CH-1784 Courtepin, Switzerland. Additional meat product samples were purchased in various stores in Berne, Switzerland.

Isolation and Characterization of Nucleic Acids

Frozen meat samples were minced with sterile surgical blades, and 0.3 g of the mince was transferred into a sterile 1.5 mL Eppendorf tube containing 430 µL of extraction buffer [10mM Tris-HCl (pH 8.0), 150mM NaCl, 2mM EDTA, and 1% (w/v) sodium dodecyl sulfate (SDS)]. Fifty microliters of 5M guanidine hydrochloride (No. G-3272, Sigma Chemicals, St. Louis, MO) and 20 µL of 20 mg/mL Proteinase K (No. 24 568, E. Merck, D-6100 Darmstadt, Germany) were added, and the mixture was mixed by inversion and incubated at 55–60°C for at least 3 h to overnight. After digestion, samples were centrifuged for 10 min at 14 500 × *g*, and 450 µL of the aqueous phase was added to 1 mL of Magic DNA purification resin (No. A 7280, Promega, Madison, WI); the mixture was mixed by gentle inversion. After the Magic DNA cleanup procedure, the purified DNA was eluted with 50 µL of 70°C water. Twenty microliters of eluate was used for estimating by agarose gel electrophoresis (12) the approximate amount of DNA and its average size; 10 µL of a 1:10 dilution was added to the 90 µL reaction mixture for PCR. As controls, genomic pig and bovine DNA were purchased from Promega and heat treated (100°C, 20 min).

Oligonucleotides

PCR primers were synthesized on an Applied Biosystems 381A synthesizer (Foster City, CA), purified over NAP-25 columns (Pharmacia, Uppsala, Sweden), and stored in the freeze-dried state at –20°C. Oligonucleotides were defined by comparison with published nucleotide sequences of the porcine (13), ovine (14), caprine (15), and bovine (16) growth hormone genes, and then, porcine-specific sequences were chosen. The oligonucleotide sequences are as follows: SW01 5′-TCAGTTTACACTCACCTGATAGCATCT-3′ (anti-sense) and SW02 5′-GGGTGGTGGAGAGGGGTGAATT-3′ (sense).

Comparison of primer sequences and their positions on the growth hormone genes are shown schematically in Figure 1. The oligonucleotides TR03 5′-TCTGCCCTAT-CAACTTTCGATGGTA-3′ (sense) and TR04 5′-AATTTGCGCGCCTGCTGCCTTCCTT-3′ (anti-sense) used for the eucaryote PCR were previously described (17).

Polymerase Chain Reaction and DNA Analysis

Amplifications of porcine DNA were carried out in a final volume of 100 µL in 0.5 mL microcentrifuge tubes containing 1× reaction buffer (Promega); 2.0 µg/mL bovine serum albumin (No. A-8022, Sigma); 1.5mM magnesium chloride;

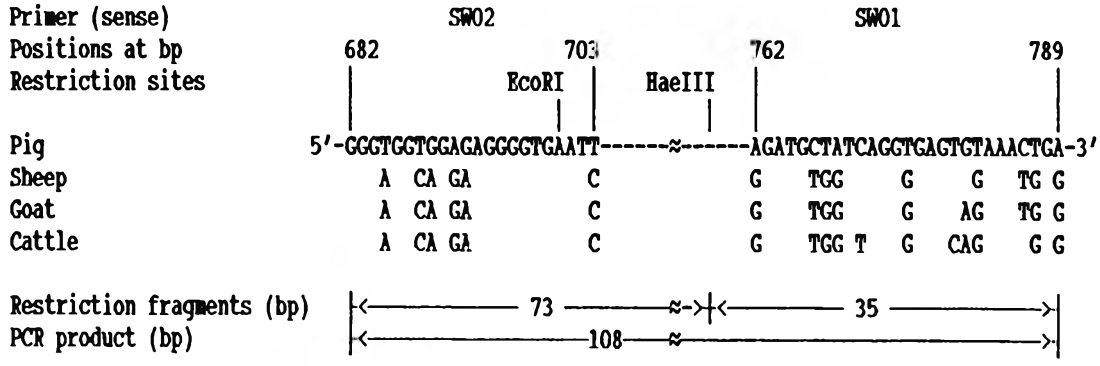

Figure 1. Comparison of primer regions of the porcine growth hormone (PGH) gene with analogous sequences from sheep, goat, and cattle. Sequence information was taken from references 13–16. Sequence positions are numbered according to reference 13.

0.2mM each of dATP, dCTP, dGTP and dTTP; 0.5μM of each primer; and 2 units of Taq DNA polymerase (Promega). A drop (80 μL) of mineral oil (Sigma) was added to cover the reaction mixture, and 30–35 cycles of amplification (first denaturation at 94°C for 3 min, 94°C for 5 s, 60°C for 30 s, 72°C for 40 s, and final extension at 72°C for 3 min) were carried out with a PHC-3 thermal cycler (Techne, Princeton, NY). Conditions for eucaryotic PCR have been described elsewhere (17). Amplification products (20 μL) were electrophoresed through 2% agarose gels (Biofinex, CH-1724 Praroman FR, Switzerland) in Tris-borate buffer (0.045M Tris-borate, 0.001M EDTA, pH 8.0) (12) and made visible by staining with ethidium bromide and UV (254 nm) transillumination. PCR products were identified by restriction enzyme digestion with *Hae*III (Boehringer, Mannheim, Germany); fragments were separated by agarose gel electrophoresis.

Immunological Analysis

A food Ouchterlony kit (The Binding Site, Birmingham, UK) was used according to the manufacturer's instructions for determining the species in mixtures (pork–beef) of raw meat samples. An ELISA (No. 902012, Cooked Meat Identification Kits, Cortecs Diagnostics, Clwyd, UK) that uses pig-specific polyclonal antibodies to heat-resistant native antigens, purified from raw skeletal muscle, was used to identify pork in mixtures (pork–beef) of heated meat samples and meat products.

Results

DNAs extracted from various animal species, pork–beef mixtures, and meat products were subjected to agarose gel electrophoresis and had DNA fragments of average size not less than 300 base pairs (bp) (even when meat had been autoclaved for up to 60 min at 121°C). According to agarose gel electrophoresis, the amount of DNA isolated was similar in all cases (data not shown). Eucaryote PCR yielding a 137-bp fragment was used to prove that this degraded DNA could serve as amplification target (complete data not shown, examples are included in Figure 2. The porcine PCR assay was demonstrated to be highly specific for porcine DNA, producing an amplification product of the expected size of 108 bp. Three pig species were tested and identified by *Hae*III digestion (data not shown). No amplification products were obtained from DNA isolated from several other species including horse, cattle, sheep, goat, chicken, and turkey (data not shown).

Heat-treated meat mixtures of pork and beef, with as little as 2% pork, were tested by porcine PCR. With 35 amplification cycles, 2% pork in beef was detected easily even when the mixture was autoclaved for 10 min at 121°C (Figure 3). These results agree with data taken from published DNA hybridization experiments using a porcine-specific DNA probe (8). Meat mixtures were also tested with commercially available immunological meat species identification kits (Table 1). Ouchterlony analysis could be used to detect 10% pork in raw meat mixtures. An ELISA for heated meat was able to confirm autoclaved pork but failed to accurately detect pork in heated meat mixtures of 20% pork in beef.

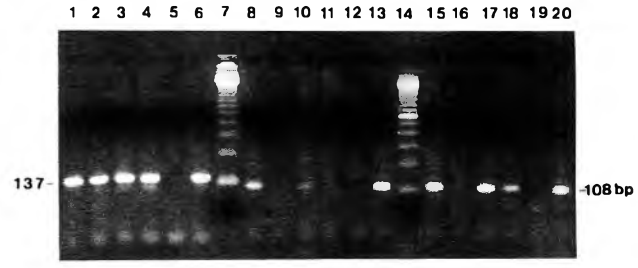

Figure 2. Agarose gel analysis of eucaryotic (137 bp) (17) and porcine-specific amplification products obtained from commercially available heat-treated meat products. Lanes 1–6, eucaryote PCR, 30 cycles; lanes 8–13, porcine PCR, 30 cycles; lanes 15–20, porcine PCR, 35 cycles. Lanes 1, 8, 15, sausage (poultry, pork); lanes 2, 9, 16, sausage (pure beef, sterilized); lanes 3, 10, 17, sausage (pure poultry); lanes 4, 11, 18 sausage (beef, sheep); lanes 5, 12, 19, negative control (no DNA); lanes 6, 13, 20, genomic pig DNA (Promega); lane 7, 123-bp ladder size standard; lane 14, 100-bp ladder size standard.

Artificial mixtures of heat-treated genomic DNA of porcine and bovine origin were used to determine the sensitivity of the porcine PCR assay. The detection limit was approximately 1% (5 ng of porcine DNA in 500 ng of total DNA) after 30 amplification cycles (Figure 4). This level of sensitivity was used to recognize fraudulent substitution but would not detect minor inadvertent contamination with pork. Increasing the cycle number to 35 allowed the detection of trace amounts (ca 0.1%) of porcine contamination (Figure 4).

PCR with DNA isolated from 15 commercially available heat-treated meat products was performed and compared with ELISAs (Table 2). Of 15 samples, 11 samples were declared to be pure beef, pure poultry, or pork-free. Porcine DNA was detected by PCR in 3 of 11 samples after 30 amplification cycles.

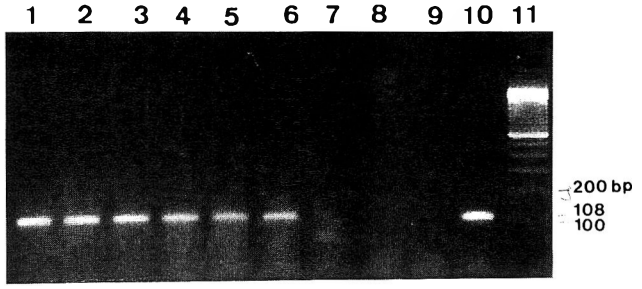

Figure 3. Agarose gel analysis of porcine-specific amplification products (108 bp) obtained after 35 PCR cycles from mixtures of pork and beef autoclaved at 121°C, 10 min. Lane 1, 100% pork; lane 2, 50% pork in beef; lane 3, 20% pork in beef; lane 4, 10% pork in beef; lane 5, 5% pork in beef; lane 6, 2% pork in beef; lane 7, 100% beef; lane 8, 100% sheep (from raw meat obtained at a butcher's shop; a very minor porcine contamination cannot be excluded); lane 9, negative control (no DNA); lane 10, porcine positive control (from raw meat); lane 11, 100 bp ladder size standard.

Table 1. Comparison of immunological (Ouchterlony and ELISA) and biomolecular (DNA hybridization and PCR) methods for species differentiation in raw and heat-treated mixtures of pork and beef[a]

Pork in beef, %	Treatment temperature, °C, and time, min	Ouchterlony[b]	ELISA[c]	DNA hybridization[d]	PCR
100	Raw	+	NT	+	+
50	Raw	+	NT	+	+
20	Raw	+	NT	+	+
10	Raw	+	NT	+	+
5	Raw	–	NT	+	+
2	Raw	–	NT	+	+
0	Raw	–	NT	–	–
100	80, 30	NA	NT	+	+
50	80, 30	NA	NT	+	+
20	80, 30	NA	NT	+	+
10	80, 30	NA	NT	+	+
5	80, 30	NA	NT	+	+
2	80, 30	NA	NT	+	+
0	80, 30	NA	NT	–	–
100	100, 20	NA	+	+	+
50	100, 20	NA	+	+	+
20	100, 20	NA	–	+	+
10	100, 20	NA	–	+	+
5	100, 20	NA	–	+	+
2	100, 20	NA	–	+	+
0	100, 20	NA	–	–	–
100	121, 10	NA	+	+	+[e]
50	121, 10	NA	+	+	+[e]
20	121, 10	NA	–	+	+[e]
10	121, 10	NA	–	+	+[e]
5	121, 10	NA	–	+	+[e]
2	121, 10	NA	–	+	+[e]
0	121, 10	NA	–	–	–[e]
100	121, 20	NA	+	NT	+
100	121, 30	NA	+	NT	+
100	121, 45	NA	+	NT	+
100	121, 60	NA	+	NT	+

[a] NA, not applicable; NT, not tested.
[b] Food Ouchterlony kit (The Binding Site).
[c] Cooked Meat Species Identification Kit (Cortecs Diagnostics).
[d] Results taken from reference 8.
[e] Results shown in Figure 3.

Two samples gave positive signals with the ELISA as well. Additional positive samples (5 of 11) were detected after 35 PCR amplification cycles (Table 2). Examples of PCR analysis for 3 food samples, which did not contain pork according to the product label, are shown in Figure 2. All gave positive results with the eucaryote PCR (lanes 2–4); after 30 cycles of porcine PCR, 1 of 3 was positive (lane 10); another was positive only after 35 cycles, implying a lower level of pork contamination (lane 18).

Discussion

Oligonucleotides used as primers for porcine-specific PCR were based on DNA sequence differences among the porcine, bovine, ovine and caprine growth hormone genes. Strategies for primer selection included searching for heterogeneous gene regions among species and superimposing the 3′-end of primers on a mismatch. PCRs with the primer pair SW01–SW02 were carried out with DNA isolated from the meat of several

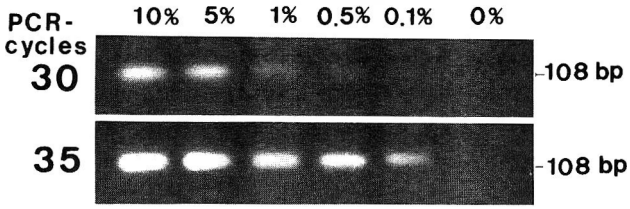

Figure 4. Agarose gel analysis of porcine-specific amplification products (108 bp) obtained from artificial mixtures of heat-treated genomic DNA of porcine and bovine origin. Five hundred nanograms of each mixture containing 10, 5, 1, 0.5, 0.1, and 0% of pig DNA in bovine DNA was subjected to 30 (top row) or 35 PCR cycles (bottom row).

pig species (two races of *Sus scrofa domestica* and wild boar) and individuals to confirm that the designed primers recognize a conserved region specific for pork. The average size of DNA fragments isolated from meat autoclaved for 10–60 min was around 300 bp. It seemed that the average size of autoclaved DNA fragments did not change significantly with longer incubation times. Therefore, the developed PCR system targeting an even shorter DNA fragment (108 bp) could be used successfully in the analysis of this highly degraded DNA. Extraction of DNA was carried out with a commercially available DNA purification resin (Magic, Promega) instead of the time-consuming standard extraction with phenol–chloroform followed by ethanol precipitation. In preliminary experiments, precipitation of degraded, small DNA fragments with ethanol was difficult and not always successful. In addition, DNA isolation with the commercial resin reliably removed PCR inhibitors such as hemoglobin. Therefore, DNA extraction could be achieved in a single step, and RNA digestion was not necessary because the resin did not bind RNA.

To develop a simple and standardized protocol, we used 300 mg of a homogeneous sample for DNA extractions from all heated products. Amounts of DNA isolated, according to agarose gel electrophoresis, were similar in all cases. Therefore an aliquot of a 10-fold dilution was used for PCR as the standard amount. The fact that eucaryote PCR yielded signals of almost equal intensity for DNAs isolated from different products indicates that no PCR inhibitors contaminated the DNA and that this approach may be useful. In general, PCR sensitivity is very high, but for meat, it seems desirable not to have a detection limit below 0.1% in order to eliminate positive results from insignificant traces of pork due to inadvertent contamination during manufacture. The procedure was therefore standardized further by limiting the amplification to 30 cycles. On the basis of experiments with pure DNA, samples with clear positive signals after 30 cycles were estimated to contain at least 5–10% pork (Figure 4). The pork contents of samples yielding a weak signal after 30 cycles and a clear signal after 35 cycles were considered to be in the range of 1–5%. Samples yielding signals only after 35 cycles were assumed to contain less than 1% pork. A clear positive signal after 30 cycles was interpreted to be adulteration (more than 5% pork; Figure 2, lane 10), whereas a positive signal only after 35 cycles is most likely due to accidental contamination during production (less than 1% pork; Figure 2, lane 18). This analysis by PCR is simple although only semiquantitative, but it can be completed within 1 working day and provides criteria for detecting intentional contamination. Preliminary results obtained by different workers in our laboratory indicate that it is not necessary to run a complete set of controls for quantification each time PCR is performed.

In contrast, DNA hybridization is time-consuming and a genomic probe is less sensitive than 2 specific oligonucleotides. ELISAs can detect species in heat-treated meat products but failed with meat mixtures containing less than 50% pork. Furthermore, cross-reactions cannot be excluded. The Ouchterlony test can be used only for raw meat mixtures containing as little as 10% pork in beef (this paper and reference 18).

PCR has advantages of specificity, sensitivity, high speed, and low cost. Assays for PCR detection of beef, soya, sheep, and goat and the identification of meat from game are in preparation. This method's analytical potential will be used to differentiate closely related species by single base-pair differences. Future applications may include the detection of transgenic domestic species, transgenic fish (19) and transgenic plants, if sequence information is available.

Acknowledgment

We thank the Federation of Migros Cooperatives, Switzerland for financial support of this work. We also thank P. Kradolfer, Migros Meat Laboratory for collaboration.

Table 2. Results of PCR assays compared with ELISA tests for a variety of meat products

Product	Species composition, declared	No. of samples	No. of samples found positive by		
			ELISA	PCR, 30 cycles	PCR, 35 cycles
Sausage	Pig, bovine	3	3	3	3
Sausage	Chicken, turkey, pig	1	1	1	1
Sausage	Bovine	3	0	0	2
Sausage	Bovine, ovine	3	0	0	3
Sausage	Chicken, turkey	4	2	3	3
Sausage	Soya, wheat	1	0	0	0

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FOOD BIOLOGICAL CONTAMINANTS

Enumeration of Total Bacteria in Raw and Pasteurized Milk by Reflectance Colorimetry: Collaborative Study

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Seven out of 9 laboratories completed a collaborative study comparing a reflectance colorimetric (RC) bioactivity monitor (Omnispec™ 4000) method to the standard plate count (SPC) method for estimation of total bacteria in raw and homogenized pasteurized milk. Each laboratory analyzed 12 different samples by the SPC method and 24 samples (12 blind duplicates) by the RC method. For the RC method RSD_r was 1.7%, and RSD_R was 4.5%. RSD_R for the SPC method was 20.8%. The method was adopted first action by AOAC INTERNATIONAL.

The standard plate count (SPC) method is used as the standard procedure for enumerating total bacteria in milk (1). Reflectance colorimetry (RC), developed as an alternative method, went through successful within-laboratory evaluations (2–5), and is currently being used for enzymatic and microbiological quality analyses of milk, meat, and cosmetic products. Results are based upon rates of bioactivity of bacterial cells measured by the reduction of triphenyl tetrazolium chloride (TTC) in nutrient broth. Thus, RC results correlate better to milk flavor changes and shelf life estimates than SPC results plate counts (3). Suggestions were made, based on results of comparative studies, to change in the RC method the lower detection limits to Log_{10} 3.000 cfu/mL with 200 µL sample volumes (3). Therefore, selective preliminary incubation (SPI) was incorporated into the method to allow increase of bacterial growth to Log_{10} 3 cfu/mL in low count samples containing Log_{10} 0.000–3.000 cfu/mL, and to ensure optimum precision. The RC method does not require solid media, sample dilutions, manual counting of plates, or extensive operator training; it only requires transferring of medium and sample into a microtiter plate well and placing the plate into the RC system. Instrument results are automatically calculated and available in approx 3–16 h, depending on microbial load.

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The recommendation was approved by the Committee on Microbiology and Extraneous Materials. The method was adopted by the Official Methods Board of the Association. See "AOAC International Official Methods Board News" (1993) *J. AOAC Int.* **76**, 33A, and "Methods Adopted First Action" (1993) *The Referee*, **17**, March issue.

Collaborative Study

Preparation of Samples

Milk sample 1 was UHT-sterilized 2% homogenized milk. Sample 2 was 2% homogenized pasteurized (HP) milk after SPI for 48 h at 7°C (3). Sample 3 was HP milk after SPI for 96 h at 7°C. Samples 4, 5, and 6 were HP milk with Log_{10} 3, 5, and 7 *Pseudomonas fluorescens* cfu/mL added, respectively. Samples 7, 8 and 12 contained the same original sample of raw whole milk 1 (RM1). Sample 7 was RM1 spiked with Log_{10} 4.632 cfu/mL, after SPI for 48 h at 7°C. Sample 8 was RM1 after SPI for 96 h at 7°C. Sample 9 contained a second raw whole milk (RM2) with Log_{10} 4.535 cfu/mL level after SPI for 48 h at 7°C. Sample 10 was RM2 after SPI for 3 h at 35°C (1). Sample 11 was a third raw whole milk (RM3), Log_{10} 4.590 cfu/mL, after SPI for 48 h at 7°C. Sample 12 was RM1 with *P. fluorescens* Log_{10} 7 cfu/mL added.

Each sample was mixed, placed in sterile vials (ca 30 mL), cooled in ice, and shipped overnight. Each laboratory received 2 sets of randomly numbered samples, labeled as sets A and B. Each set also contained a temperature check vial.

Analysis of Samples

Seven laboratories (23 analysts) participated in this study. Each laboratory was provided with detailed instructions, report sheets, computer software report discs, and materials for RC analyses. Each laboratory had an Omnispec 4000 reflectance colorimeter, and used the same instrument for each analysis. Collaborators were instructed to analyze the samples the day after receipt. Laboratories not routinely performing the SPC procedure were provided the supplies necessary to run the analyses. The SPC was run according to standard methods (1).

Table 993.11A. Method performance for bacterial counts in raw and pasteurized milk by reflectance colorimetric (RC) method

			RC				
Product[a]	Incubation	Spike/mL[b]	Mean, Log_{10} cts/mL[c]	s_r	s_R	RSD_r, %	RSD_R, %
UHT, 2%			<3.000				
HP1, 2%	48 h, 7°		4.825	0.131	0.473	2.71	9.80
HP1, 2%	96 h, 7°		6.941	0.130	0.337	1.87	4.85
HP1, 2%		Log_{10} 3	4.867	0.130	0.459	2.67	9.43
HP1, 2%		Log_{10} 5	6.612	0.136	0.299	2.05	4.52
HP1, 2%		Log_{10} 7	6.410	0.144	0.195	2.24	3.04
RW1	48 h, 7°		7.468	0.085	0.191	1.14	2.56
RW2	48 h, 7°		7.391	0.055	0.211	0.74	2.85
RW3	48 h, 7°		7.436	0.099	0.248	1.33	3.33
RW1	96 h, 7°		7.690	0.080	0.157	1.04	2.04
RW2	3 h, 35°		7.574	0.076	0.133	1.00	1.76
RW1		Log_{10} 7	6.558	0.146	0.259	2.23	3.95
Mean			6.706	0.110	0.269	1.73	4.41

[a] UHT, 2% = 2% milk treated at ultra high temperature; HP1, 2% = 2% homogenized pasteurized milk, sample 1; RW1 = raw whole milk, sample 1; RW2 = raw whole milk, sample 2; RW3 = raw whole milk, sample 3.
[b] Sample spiked with *Pseudomonas fluorescens*.
[c] Color detection time (CDT) values converted to Log_{10} count/mL using calibration curve calculated from **C***(e)*: Y = 8.8674 – 0.3836X, (R^2= 0.9850, RMSE = 0.1870).

993.11 Bacterial Counts in Raw and Pasteurized Milk—Reflectance Colorimetric Method (Omnispec) First Action 1993

(Applicable to enumeration of $\geq 10^{4.5}$ cfu/mL bacteria in milk)
Method Performance:

See Tables **993.11A** and **993.11B** for method performance data.

A. Principle

Bacteria present in milk samples reduce triphenyl tetrazolium chloride (TTC) dye in nutrient medium during incubation. Reflectance colorimeter analyzes samples at regular inter-

Table 993.11B. Method performance for bacterial counts in raw and pasteurized milk by standard plate count (SPC) method

			SPC		
Product[a]	Incubation	Spike/mL[b]	Mean, Log_{10} cts/mL[c]	s_R	RSD_R, %
UHT, 2%			<3.000		
HP1, 2%	48 h, 7°		4.308	1.910	43.72
HP1, 2%	96 h, 7°		5.988	1.837	30.68
HP1, 2%		Log_{10} 3	5.784	1.959	33.88
HP1, 2%		Log_{10} 5	5.987	2.137	35.69
HP1, 2%		Log_{10} 7	7.317	1.160	15.85
RW1	48 h, 7°		7.482	0.570	7.62
RW2	48 h, 7°		7.442	0.598	8.04
RW3	48 h, 7°		7.543	0.898	11.90
RW1	96 h, 7°		7.684	0.752	9.78
RW2	3 h, 35°		7.577	0.816	10.77
RW1		Log_{10} 7	7.156	0.888	12.40
Mean			6.756	1.23	20.03

[a] UHT, 2% = 2% milk treated at ultra high temperature; HP1, 2% = 2% homogenized pasteurized milk, sample 1; RW1 = raw whole milk, sample 1; RW2 = raw whole milk, sample 2; RW3 = raw whole milk, sample 3.
[b] Sample spiked with *Pseudomonas fluorescens*.
[c] Color detection time (CDT) values converted to Log_{10} count/mL using calibration curve calculated from **C***(e)*: Y = 8.8674 – 0.3836X, (R^2= 0.9850, RMSE = 0.1870).

vals for color changes; when soluble colorless TTC converts to insoluble red form, colorimeter estimates bacterial count. Calibration values in computer software allow estimation of bacterial count in original sample on basis of color detection time (CDT), i.e., incubation time before color change.

B. Apparatus and Reagent

(a) *Reflectance colorimeter (RC) system.*—Equipped with incubator, colorimeter, and computer with Omnispec software installed (Omnispec 4000, Wescor, Inc., Logan, UT, is suitable source).

(b) *Medium.*—Containing pancreatic digest of casein, 2.5%; yeast extract, 1.25%; glucose, 0.5%; and triphenyltetrazolium chloride, 0.04% in H_2O. Sterilize (0.2 μm filter) 10 mL/tube (Medium "A," Wescor, Inc., is suitable).

(c) *Microtiter plates/lids/sealing tape.—(1)* Flat bottom microtiter plates containing 96 wells, with lids. *(2)* Plate sealing tape or invisible plastic tape.

(d) *Pipettors/tips.—(1) Adjustable pipettor.*—50–200 μL. *(2) Fixed Volume Pipettor.*—200 μL. *(3) Sterile tips.*—To fit *(1)* and *(2).*

(e) *Reagent reservoirs.*—Sterile reagent reservoirs, plastic (Costar # 4870, Baxter Healthcare Corp., is suitable source).

C. Computer-Based Calibration and Control

(a) *Colorimeter calibration.*—At computer power up, insert instrument calibration plate (white color standard). Press enter and instrument measures white color standard and calibrates color scales in instrument.

(b) *Reflectance colorimeter system start up.*—Using software command "Add a Test," program the following into computer software: Test Name: RC Plate Count; Color Parameter: a*; Color Endpoint: 4.00 Baseline [+ -]; Values Increase to Endpoint? Y; Take Sample at Startup Time? N; Test Interval Time: 0 h 30 min; Number of Intervals: 32; Y Intercept: 8.8674; Line Slope:–0.3836 (intercept and slope values may vary with market and be changed after local recalibration); Reporting Limits: Insert Log values for local standards (i.e., If local lower standard is 10 000 cfu/mL, then insert 4.000 in -NEG- box (Will display in green on monitor and with normal type on printout.). If margin values are 10 000–50 000 cfu/mL, insert 4.700 in :MRGN: box (Will display in yellow on monitor and with bold type on printout.). The +POS+ box will automatically show Log 4.700 and will program all estimates above Log 4.700 to be displayed in red on monitor and with underlined bold type on printout.

(c) *Recalibration of SPC correlation.*—Split ≥12 samples raw or pasteurized milk into 2 samples, >30 mL each. Store 1 set 48 h at 0.0–4.4°. Incubate other set at selective preliminary incubation (SPI) time and temperature suitable to allow Log 3 increase in cfu/mL (48 h at 7° for psychrotrophs or 3–4 h at 35° for mesophilic organisms). Analyze samples using reference pour plate technique, **966.23C**. Samples without SPI will range ca 3 logs lower than SPI samples. Measured values will depend upon initial bacterial levels. Analyze duplicate samples in colorimeter. Export RC data into statistical program. Input SPC data and calculate correlation coefficient and regression line values. Program new intercept and line slope values into "Edit a Test" program, *see* (b).

Table 1. Collaborative study results for *Pseudomonas fluorescens* (Log_{10} counts/mL) in milk samples using standard plate count (SPC) method

		Laboratory					
Sample	AR[a]	1	2	3	4	5	6
1	<3.000	<3.000	<3.000	<3.000	<3.000	<3.000	<3.000
2	4.573	4.000	4.255	2.954	4.204	2.301	8.292
3	7.198	5.785	6.568	2.602	5.380	5.835	8.546
4	4.899	4.800	8.869	3.835	4.447	5.398	8.240
5	6.835	6.401	8.322	2.954	6.778	3.040	7.581
6	6.457	5.806	8.544	8.462	6.322	7.204	8.426
7	7.512	7.000	8.642	7.431	7.322	6.903	7.562
8	7.362	7.267	8.964	6.954	7.462	7.255	8.526
9	7.415	7.114	8.748	6.954	7.415	7.204	7.243
10	7.380	6.485	8.881	7.301	7.431	7.114	8.446
11	7.470	6.611	8.892	7.000	7.301	6.845	8.683
12	6.649	6.447	8.556	6.903	6.362	6.903	8.274

[a] Authors' results.

Table 2. Collaborative study results for *Pseudomonas fluorescens* (Log_{10} count/mL) of blind duplicates (A and B) using reflectance colorimetric (RC) method

	Laboratory					
Sample	1	2	3	4	5	6
1A	<3.000	<3.000	<3.000	<3.000	<3.000	<3.000
1B	<3.000	<3.000	<3.000	<3.000	<3.000	<3.000
2A	5.470	4.274	4.637	4.559	5.188	5.141
2B	5.245	3.902	4.703	4.559	5.082	5.141
3A	7.439	7.038	7.063	6.343	7.195	6.957
3B	7.245	6.666	7.013	6.343	7.038	6.957
4A	5.357	4.221	4.969	4.559	5.188	5.141
4B	5.301	3.955	5.138	4.559	4.872	5.141
5A	6.982	6.340	6.897	6.437	6.772	6.637
5B	7.041	5.971	6.738	6.437	6.562	6.531
6A	6.475	6.146	6.678	6.343	6.509	6.531
6B	6.421	6.130	6.681	6.343	6.616	6.049
7A	7.608	7.517	7.780	7.282	7.301	7.401
7B	7.495	7.570	7.730	7.282	7.354	7.295
8A	7.717	7.677	7.799	7.752	7.639	7.454
8B	7.661	7.730	8.058	7.752	7.583	7.454
9A	7.439	7.304	7.889	7.282	7.301	7.295
9B	7.323	7.304	7.783	7.282	7.195	7.295
10A	7.608	7.570	7.780	7.689	7.583	7.295
10B	7.495	7.517	7.673	7.752	7.473	7.454
11A	7.439	7.464	7.946	7.282	7.248	7.295
11B	7.439	7.464	7.839	7.439	7.354	7.029
12A	6.475	6.130	7.116	6.343	6.403	6.637
12B	6.475	6.562	6.957	6.343	6.616	6.637

Table 3. Comparison of R^2 values of regresion between the authors' (AR) and collaborators' SPC and RC data

	Laboratory						
Comparison	AR	1	2	3	4	5	6
AR SPC/SPC	1.0000	0.9235	0.6414	0.3509	0.8857	0.5371	0.4143
AR SPC/RC	1.0000	0.9626	0.9673	0.9872	0.9155	0.9710	0.9813

(**d**) *System uitability.*—For colored substances, make repeated measurements to ensure reproducible color measurements (*Note*: absolute color values are not critical in this method).

(**e**) *Calculation of cfu/mL.*—Correlation of cfu/mL with detection time is approximated by linear regression with a negative slope. cfu/mL is automatically calculated from detection time as follows:

$$\text{cfu/mL} = \text{intercept} + (\text{slope} \times \text{detection time})$$

Using values entered in *(b)*, equation becomes:

$$\text{cfu/mL} = 8.8767 - 0.3836(\text{detection time})$$

D. Sample Preparation

Shake samples 25× in 30 cm arc for 7 s. Let foam subside 30 s before sampling.

E. Determination

Pipet 50 µL medium into microtiter plate wells for each sample tested. Pipet 200 µL each sample into separate wells and mix by refilling and discharging contents 3×. Prepare in duplicate if desired. (If samples become contaminated, mark contaminated well by adding drop of India ink into well; resample into uncontaminated well.)

Remove backing from sealing tape and apply to microtiter plate, sealing tightly around all wells. Alternatively, replace lid and seal between lid and plate using invisible plastic tape (taking care to avoid tilting plate and contaminating wells).

Check that incubator is at 30 ± 1°. Start "Begin A Test" following computer software instructions. Results are displayed on monitor as CDTs are recorded. After 16 h, results are printed out, either in number (eg., 1.8×10^4) or in Log_{10} format (eg., 4.255), as selected.

Table 4. Analysis of variance of SPC method

Source	DF[a]	SS[b]	MS[c]	F[d]	P[e]
Sample	11	282.428	25.675	16.81	0.0001
Error	48	73.320	1.527		
Total	59	355.748			

[a] DF = degrees of freedom.
[b] SS = sum of squares.
[c] MS = mean square.
[d] F = F value.
[e] P = significance.

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Results and Discussion

The RC system permitted simultaneous analysis of 4 assays; 7 instruments were used by 23 analysts. Data from 1 laboratory were not used because samples were not analyzed for 3 days after receipt, and the analyses were not complete (Table 1). Another laboratory could not participate in the study as scheduled. The sample volumes used by 1 laboratory were too high. Two laboratories returned data showing contamination of the wells containing UHT milk. Data from 2 laboratories were excluded due to SPC equipment failure. After removal of invalid data, 17 data sets were used for statistical analysis. Precision statistics were estimated using the 6 laboratories (17 analysts) that performed the SPC analyses. No outlier points were detected.

The data in Table 2 were converted from CDT values to Log_{10} count/mL using a calibration curve calculated from the authors (AR) SPC and RC data (Set A); $Y = 8.8674 - 0.3836X$ ($R^2 = 0.9850$; RMSE = 0.1870; Mean = 6.3958; N = 12.)

R^2 values among the 6 laboratories were compared to the AR SPC data in Table 3. The RC method estimated AR SPC data better than the SPC method.

Precision statistics are summarized in Table 4. The RSD_R was higher than reported in previous studies (6) probably because only 3 laboratories routinely perform the SPC analyses. Data from these 3 laboratories produced $RSD_R = 6.7\%$, while the remaining 4 laboratories produced $RSD_R = 25.6\%$. Laboratory 6 reported contamination of dilution blanks; the SPC data was not used in the statistical analysis.

Table 5. Analysis of variance of RC method for 6 laboratories

Source	DF[a]	SS[b]	MS[c]	F[d]	P[e]
Sample	11	275.914	25.083	2169.03	0.0001
Lab	5	3.374	0.675	58.35	0.0001
Sample × lab	55	5.899	0.107	9.27	0.0001
Error	72	0.833	0.012		
Total	143	286.020			

[a] DF = degrees of freedom.
[b] SS = sum of squares.
[c] MS = mean square.
[d] F = F value.
[e] P = significance.

Table 6. Analysis of variance of RC method for 17 analysts

Source	DF[a]	SS[b]	MS[c]	F[d]	P[e]
Sample	11	710.768	64.615	1250.34	0.0001
Lab	5	40.784	8.157	157.84	0.0001
Opr/lab	11	4.309	0.392	7.58	0.0001
Sample × lab	55	24.209	0.440	7.27	0.0001
Sample × opr/lab	121	5.616	0.046	0.90	0.7401
Error	204	10.542	0.052		
Total	407	811.600			

[a] DF = degrees of freedom.
[b] SS = sum of squares.
[c] MS = mean square.
[d] F = F value.
[e] P = significance.

For the RC method RSD_r was 1.7% and RSD_R was 4.5% calculated from the data obtained from 6 laboratories (Table 5). These values were within the ranges reported for the SPC method (RSD_r = 1.1–5.1 and RSD_R = 1.5–15.3%) (6). The values for the 17 analysts were 3.9 and 5.9%, respectively, and were also within this range (Table 6). They demonstrate the ability of untrained analysts to obtain precision values comparable to those who are trained in SPC procedure.

Comparative studies (3) have provided a lower limit of Log_{10} 3.000 cfu/mL for RC when using 200 µL samples. SPI (3) was used in sample preparation in this study in order to provide estimates down to Log_{10} 0.000 cfu/mL. SPI for 3 h at 35°C is routine for impedance bioactivity monitors for coliforms (1). Samples 2,7,9, and 10 went through SPI for 48 h at 7°C to achieve a mean Log_{10} increase of 2.88 cfu/mL. For such samples the calibration curve intercept would be reduced by the magnitude of the SPI increase from 8.8674 to 2.88 (reduction by 5.9874 mean Log_{10} count/mL.) This way the instrument limit of detection is reduced from Log_{10} 3 to Log_{10} 0 cfu/mL and is useful for estimation of extremely low microbial loads.

Recommendation

It is recommended that the reflectance colorimetric method for the enumeration of total bacteria in raw and pasteurized milk be adopted first action.

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FOOD CHEMICAL CONTAMINANTS

Determination of Deoxynivalenol in 1991 U.S. Winter and Spring Wheat by High-Performance Thin-Layer Chromatography

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A thin-layer chromatographic (TLC) method was modified for determination of deoxynivalenol (DON) in 1991 U.S. winter and spring wheat. After extraction with acetonitrile–water (84 + 16) and cleanup on a charcoal–alumina–Celite (7 + 5 + 3) column, acetonitrile was used instead of ethyl acetate to transfer the concentrated extract containing DON. After the extract was evaporated to dryness, the residue was dissolved in methanol and an aliquot was spotted on a high-performance TLC plate. After development with chloroform–acetone–2-propanol (8 + 1 + 1), the plates were sprayed with aluminum chloride solution and heated; DON was quantitated by fluorodensitometry. Average recoveries of DON added to duplicate test portions of wheat at 200, 400, and 800 ng/g were 83, 82, and 72%, respectively. The detection limit was 40 ng/g. The method was applied to 81 test samples of spring and winter wheat. The wheat contained DON levels that ranged from nondetectable to 9330 ng/g (average 1570 ng/g). The results indicate that DON levels were higher in wheat from Missouri, North Dakota, and Tennessee than in wheat from 7 other states. The identity of DON, which was isolated from 21 of the extracts by preparatory TLC, was confirmed by gas chromatography/mass spectrometry in all 21 test samples.

Deoxynivalenol (DON, vomitoxin, 3,7,15-trihydroxy-12,13-epoxytrichothec-9-en-8-one) is one of the toxic trichothecenes produced by various species of *Fusarium* and other fungal genera that under certain climatic conditions invade grains such as corn, barley, and wheat in field and storage (1). DON toxicity symptoms include nausea, vomiting, visual disturbances, chills, vertigo, and food refusal with some farm animals (2). Several countries routinely monitor for DON presence. First identified in Japan in 1972, DON has since been found in grains in other parts of the world. In North America, DON occasionally has been a problem in wheat products. Within the trichothecenes toxin group, only DON has been found in significant amounts in North America.

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In the summer of 1991, both winter and spring wheat crops were reported to have a greater than normal incidence of pink scab caused by *Fusarium graminearum*. This infection was associated with increased rainfall in the wheat growing areas. Because scabby wheat is often contaminated with DON (1), the U.S. Food and Drug Administration (FDA) collected wheat samples and analyzed them for DON.

Several analytical methods have been developed for determining DON (1–8). One method uses gas chromatography/mass spectrometry (GC/MS), which requires considerable time and investment in equipment (3). A thin-layer chromatographic (TLC) procedure developed by Trucksess et al. (4) gave DON recoveries >80%. The TLC procedure, which was extensively evaluated in several laboratories, was used to determine DON in wheat from the 1982 crop year (1) and was adopted as official first action by AOAC after collaborative study (5).

The method described in this paper, which incorporates several improvements in the Trucksess et al. method (4), includes rapid, efficient extraction and cleanup procedures combined with high-performance TLC as the determinative step. The modified method was applied to the analysis of 1991 spring and winter wheat collected in the United States. The chemical identity of DON in 21 of the positive test samples was confirmed by a GC/MS system.

METHOD

Reagents

(a) *Adsorbents for cleanup.*—Activated charcoal, Darco G-60 (J.T. Baker Chemical Co., Phillipsburg, NJ 18865); neutral alumina, chromatographic grade, 80–200 mesh (No. AX0612, E.M. Science, Cherry Hill, NJ 08034); and diatomaceous earth, acid-washed Celite 545 (Johns-Manville Products Corp., Denver, CO 80217). Prepare cleanup column as follows: Place 0.1 g Celite in bottom of chromatographic tube. Thoroughly mix 0.7 g charcoal, 0.5 g alumina, and 0.3 g Celite in 50 mL beaker with spatula. Add mixture to chromatographic tube and lightly tap tube to settle packing. Add ball of glass wool on top and apply suction to compress column packing.

(b) *Aluminum chloride spray solution.*—Dissolve 20 g $AlCl_3 \cdot 6H_2O$ in 100 mL methanol–water (1 + 1).

(c) *DON standard solution.*—25 μg/mL in methanol. DON is available from Robert Eppley, FDA, Washington, DC 20204, and commercially from several sources.

(d) *Solvents.*—Acetonitrile; methanol; acetone–chloroform (1 + 1); extraction and wash: acetonitrile–water (84 + 16); TLC development: chloroform–acetone–2-propanol (8 + 1 + 1); derivatization: silylation grade acetonitrile (Pierce Chemical Co., Rockford, IL 61105).

(e) *Derivatizing reagent for GC/MS.*—*N,O*-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) (Pierce Chemical Co.).

Apparatus

(a) *Mill.*—Retsch SR3 (Retsch, 5657 Haan, Germany).

(b) *Chromatographic tube.*—Baker 19 SPE filtration system, packed with dual 2 μm frits, 6 mL (No. 7121-6, J.T. Baker Chemical Co.).

(c) *Vacuum apparatus.*—J.T. Baker extraction system.

(d) *High-performance TLC plates.*—20 × 10 cm Linear-K High Performance (LHP-K) silica gel (Cat. No. 4805-710, Whatman Inc., Clifton, NJ 07014).

(e) *Densitometer.*—Camag TLC/HPTLC scanner, monochromator set at 366 nm, 110 V (Cat. No. 76511, Applied Analytical Industries, Inc., Wilmington, NC 28405).

(f) *Integrator.*—SP 4100 (Spectra-Physics, San Jose, CA 95134).

(g) *Preparatory TLC plates.*—20 × 20 cm silica gel (No. 5763, Merck & Co., Rahway, NJ 07065).

(h) *Syringeless filter for organic solvent.*—UniPrep, with 0.45 μm polytetrafluoroethylene membrane and glass microfiber prefilter (Genex Corp., Gaithersburg, MD 20877).

(i) *Quadrupole mass spectrometer.*—Finnigan Model 4023 (San Jose, CA 95134), equipped with a Finnigan gas chromatograph. The instrument was operated in the positive ion electron ionization mode.

Extraction

Grind each test sample (1 kg) to pass 2 mm sieve. Weigh 50 g ground wheat into 500 mL glass-stoppered flask, add 200 mL acetonitrile–water (84 + 16), seal flask with masking tape, and shake 30 min, using wrist-action shaker at fast rate. Filter mixture and collect 20 mL filtrate in 25 mL graduated cylinder.

Column Chromatography

Attach charcoal–alumina–Celite (7 + 5 + 3) cleanup column to vacuum apparatus and place beaker in chamber to collect eluted solvent. Transfer 20 mL filtrate to column (2–3 mL/min flow rate). As last of solution enters packed column bed, rinse cylinder with 10 mL acetonitrile–water (84 + 16) and add rinsings to column. Continue vacuum until flow stops. Remove beaker and evaporate solvent under stream of nitrogen on steam bath until ca 3 mL solution remains. (*Note*: Do not evaporate to dryness.) Transfer concentrated extract to 2 dram vial. Wash beaker with three 1 mL portions of acetonitrile and combine washes in vial. Evaporate to dryness under stream of nitrogen on steam bath.

Thin-Layer Chromatography

Add 100 μL methanol to vial and dissolve residue by using vortex mixer ca 1 min. Spot 5 μL partially purified extract twice and spot 2, 5, and 10 μL DON standard solution (25 μg/mL) on high-performance TLC plate; place spots exactly 1 cm apart and 1.8 cm from bottom of plate. Develop plate in closed, unequilibrated tank with chloroform–acetone–2-propanol (8 + 1 + 1) to height of ca 9.0 cm. Remove plate, air-dry 10 min, and spray evenly with $AlCl_3$ solution. Heat plate in 120°C convection oven 7 min. Remove plate from oven and examine under longwave (365 nm) UV light. DON appears as a blue fluorescent spot with R_f = 0.78. Compare spots from test extract with those from standard. Scan spots with densitometer from top to bottom, parallel to direction of development. Densitometric results must be confirmed by visual inspection. DON spots from test extract and standard should be the same fluorescent color. Response is linear relative to DON concentrations of 10–250 ng. Highly concentrated extracts, containing >1000 ng DON/g, should be diluted and the TLC redone. Evaporate extract remaining in vial to dryness and save for TLC-GC/MS confirmation.

Calculations

Calculate concentration of DON in test sample, using the following equation:

$$\text{ng/g (ppb)} = S \times \frac{C}{X} \times \frac{V}{W}$$

where S = densitometric response for DON in test extract; C = concentration of DON in standard solution; X = densitometric response for DON standard corresponding to volume of test extract spotted; V = final volume of test extract, 100 μL; and W = weight of test sample represented by final volume of test extract, 5 g.

Mass Spectrometric Confirmation

Preparatory TLC.—The identity of DON in some test samples should be confirmed by GC/MS (3). Dissolve residue remaining from TLC analysis in 100 μL methanol. On one regular TLC plate, spot DON standard solution 4 times (10 μL each). On another plate spot entire remaining test extract (one extract per plate). The standard plate is used as a reference to locate DON on test extract plate. (*Caution*: Do not spray test extract plate because intact DON is required for GC/MS confirmation.) Develop both plates at the same time, in the same closed and unequilibrated tank with chloroform–acetone–2-propanol (8 + 1 + 1). Take both plates from tank at the same time and air-dry 10 min. Spray standard plate only with $AlCl_3$ solution and heat 7 min at 120°C. DON is identified as a blue fluorescent spot. Remove silica gel from test extract plate at same R_f as that of DON standard. Mix silica gel obtained from scraped plate with acetone–chloroform (1 + 1) and filter mixture through Uniprep syringeless filter. Evaporate filtrate to dryness under stream of nitrogen and dissolve residue in 100 μL methanol in 2 dram vial. Spot 10 μL test solution and

2, 5, and 10 μL of DON standard solution on LHP-K plate. Develop, spray with $AlCl_3$ solution, and heat plate. Measure fluorescence of spot with densitometer. Calculate amount of DON remaining after preparatory TLC. Evaporate remaining test solution to dryness and use for derivatization procedure for GC/MS confirmation.

Derivatization procedure.—Add 200 μL silylation grade acetonitrile, followed by 50 μL BSTFA, to dry residue film in vial. Cap vial, shake, and heat at 70°C for 1 h. After solution cools, evaporate to dryness under stream of dry nitrogen. Dissolve resulting film in 50 μL dry acetonitrile and analyze solution by GC/MS.

GC conditions.—Splitless mode; 15 m × 0.2 mm id HP-1 (methyl silicon) capillary column with 0.1 μm film thickness; helium 35 cm/s; injector and transfer line temperature 260°C; initial oven temperature 160°C, program at 20°C/min to 260°C, hold for 5 min. Inject ca 1 μL test solution.

MS conditions.—Emission current 0.47 mA, electron energy 23 eV, scan from 60 to 650 amu at rate of 1.05 s/scan. Acquisition is started when GC temperature reaches 200°C.

Results and Discussion

The TLC method of Trucksess et al. (4) was modified as described for determination of DON in 1991 U.S. winter and spring wheat. Acetonitrile instead of ethyl acetate was used in the cleanup step to transfer the concentrated extract containing DON because of problems in dissolving the residue obtained in the original method. After the extract was evaporated to dryness, the residue was dissolved in methanol instead of acetonitrile–chloroform, and aliquots were spotted on a high-performance TLC plate. Instead of impregnating the plate with $AlCl_3$ before analysis, the plate was sprayed with $AlCl_3$ solution and heated after development.

With the modified method, 8–16 test samples can be analyzed in 1 day. The average recoveries of DON added to duplicate test portions of wheat at 200, 400, and 800 ng/g levels were 83, 82, and 72%, respectively. The detection limit was 40 ng/g. To evaluate the method, the preparatory TLC-GC/MS confirmatory procedure was tried on 4 naturally contaminated test samples previously found to contain 0, 915, 1830, and 3040 ng DON/g wheat. The identity of DON in all 3 positive test samples was confirmed and no DON was found in the blank extract. The chemical identity of DON in 21 of the positive test samples analyzed in this study was also confirmed by the preparatory TLC-GC/MS confirmatory procedure.

The Federal Grain Inspection Service of the U.S. Department of Agriculture (USDA) supplied 81 samples of 1991 wheat. Spring wheat was obtained from Idaho, Minnesota, North Dakota, and South Dakota. Winter wheat was obtained from the other 6 states. A summary of the results from the analyses is shown in Table 1. The wheat contained DON levels that ranged from nondetectable to 9330 ng/g (average 1570 ng/g). The results indicate higher amounts of DON present in wheat from Missouri, North Dakota, and Tennessee compared with the other states. This finding may have been caused by increased rainfall experienced in these states during the 1991 growing season and the concomitant increase in the amounts of scabby wheat reported. The average amount of DON present in wheat in years of normal rainfall is not known.

Table 1. Deoxynivalenol in 1991 U.S. wheat[a]

State	No. of test samples analyzed	DON found, ng/g[b]	
		Av.	Concn range
Idaho	6	29	ND-90
Maryland	6	45	ND-86
Minnesota	1	1376	—
Missouri	8	5203	2583-9330
North Carolina	12	1164	220-2110
North Dakota	6	5133	1596-6303
South Carolina	6	170	41-306
South Dakota	23	852	ND-2605
Tennessee	6	2404	1570-4751
Virginia	7	535	199-1577
United States	81	1570	ND-9330

[a] The Federal Grain Inspection Service of the U.S. Department of Agriculture supplied the wheat samples.
[b] ND = Nondetectable. Detection limit = 40 ng/g.

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FOOD CHEMICAL CONTAMINANTS

Detoxification of Citrinin and Ochratoxin A by Hydrogen Peroxide

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The effects of hydrogen peroxide on citrinin and ochratoxin A toxicity were examined using HeLa cells. The citrinin was completely detoxified by prior incubation with 0.05% hydrogen peroxide for 30 min at room temperature, and the toxic compound(s) that resulted from heating citrinin at 100°C were also detoxified upon reheating it with hydrogen peroxide. On the other hand, ochratoxin A was not detoxified by hydrogen peroxide at room temperature, but its toxicity was reduced by heating ochratoxin A with hydrogen peroxide under alkaline conditions.

The mycotoxins citrinin and ochratoxin A sometimes occur together in naturally contaminated food and feed (1–3). Citrinin is chemically formulated as a quinone methide and ochratoxin A is a chlorinated dihydroisocoumarin linked through the 7-carboxyl group to L-phenylalanine by an amide bond. Both are potent nephrotoxins and are toxic to many animal species (4–7).

To decompose and detoxify these mycotoxins, heating and chemical treatments were investigated. Heating processes can be an effective way to degrade citrinin (8–10). Citrinin can be decomposed and detoxified by heating at 175°C under dry conditions. Kitabatake et al. (11) reported that the detoxification temperature for citrinin could be reduced by 35°C when it was heated in the presence of a small amount of water, i.e., moist conditions. However, Kitabatake et al. (11) also found that the heating of citrinin under moist conditions led to the occurrence of additional toxic compounds. One of these toxic compounds, citrinin H1, which is more toxic than citrinin, has been purified and its structure has already been identified (12). Citrinin H1 and other toxic compounds are formed with prolonged heating, even at 100°C or lower temperatures (13). Although heating is an effective method to destroy citrinin, it may elicit additional toxic compounds, depending on the heating conditions. An effective method for detoxifying not only citrinin but also other heat-induced toxins formed from citrinin should be sought.

Chemical treatments to detoxify citrinin have not been extensively investigated. Citrinin appears to be unstable in acid (14) and is thermolabile in either acid or alkaline solutions (15). An alkaline (2% ammonia) solution destroys citrinin within 6 days at 30°C (16). Madsen et al. (17) reported that citrinin was decomposed in barley by 0.5% NaOH at 100–110°C. The effects of other chemicals on citrinin are not well known.

Ochratoxin A is a more toxic and stable compound than citrinin. Heating cannot easily detoxify it (18). The ochratoxin A molecule possesses both an amide bond and a lactone group. These sites can be attacked during chemical and physical treatments (19). Chelkowski et al. (16) reported that the treatment of ochratoxin A-contaminated grain with a solution of 2% ammonia reduced the ochratoxin A concentration to undetectable levels, and the compounds resulting from this decomposition were much less toxic than ochratoxin A. However, Paster et al. (20) found that purified ochratoxin A is stable in the presence of 2% and 5% ammonia at 4°C and is stable for over 30 days at 28°C. While treatment with alkali is also an effective way to detoxify ochratoxin A (21), a high concentration of alkalinity is needed to detoxify it. Enzyme treatment might also be a possible method to detoxify ochratoxin A. Pitout (22) reported that ochratoxin A was hydrolyzed by carboxypeptidase to ochratoxin α, which is nontoxic. Although enzyme treatment could be effective and mild, it is difficult to apply to the actual food or feed from an economical aspect. Therefore, a new and improved method for detoxification of ochratoxin A is required.

Oxidizing and reducing agents are presently used to modify, sanitize, and improve food properties. Hydrogen peroxide is one of these chemicals and is known to be effective in destroying aflatoxin, zearalenone, and deoxynivalenol (23). The reduction and detoxification of aflatoxin B_1 in peanut meal has been reported using a combination of hydrogen peroxide, alkaline, and heat treatments (24).

Mycotoxins exist along with various other compounds in food and feed. These compounds may interact with mycotoxins to influence their toxicity; heating in particular may promote this effect. On the other hand, living organisms have systems to detoxify toxic compounds ingested with food or feed. One of these detoxification systems involves the liver, where lipophilic compounds are hydroxylated and/or hydrated and then excreted. Glycine, glutamine, taurine, cystein, glucuronic acid, etc., form conjugates with such toxic compounds. Therefore, we attempted to mix and heat citrinin with such compounds and then to examine its toxicity.

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The purpose of this study was to find a detoxification method for citrinin, for the toxic compounds (including citrinin H1) formed from citrinin upon mild heating, and for ochratoxin A, using chemical and heat treatments that could be applicable in practical use.

METHOD

Reagents

(a) *Ochratoxin A.*—Benzene-free ochratoxin A (Makor Chemicals, Ltd., Jerusalem, Israel).

(b) *Chloroform.*—Spectrophotometric grade (Nacalai Tesque, Inc., Kyoto, Japan).

(c) *Ethanol.*—Spectrophotometric grade (Nacalai Tesque, Inc., Kyoto, Japan).

(d) *Dulbecco's modified Eagle's medium.*—(Sigma Chemical Co., St. Louis, MO).

(e) *3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT).*—Sigma Chemical Co.

(f) *Trypsin.*—Gibco/BRL Life Technologies, Inc., Gaithersburg, MD.

(g) *Isopropanol.*—(Wako Pure Chemical Industries, Ltd., Osaka, Japan).

(h) *Fetal bovine serum (FBS).*—(Whittaker Bioproducts, Inc., Walkersville, MD).

(i) *Hydrogen peroxide (H_2O_2).*—(Santoku Chemical Industries Co., Ltd., Miyagi, Japan).

(j) *HeLa cell line.*—(Dainippon Pharmaceutical Co., Ltd. Osaka, Japan).

Apparatus

(a) *Silica gel column.*—Wakagel C-300 (Wako, Osaka, Japan)

(b) *Glass vial.*—1 mL (V-1A, Nichiden Rika Glass Co., Ltd., Tokyo, Japan).

(c) *UV-Vis spectrophotometer.*—Shimadzu recording spectrophotometer (UV-160A, Shimadzu Co., Kyoto, Japan.)

(d) *Microplate reader.*—Tosoh MPRA-4 (Tokyo, Japan).

(e) *96-well microplate.*—A/S Nunc, Kamstrup, Denmark.

(f) *Sonicator.*—Heat Systems Inc., Farmingdale, NY.

Preparation of Citrinin

Citrinin was prepared in the laboratory from the toxigenic strain of *Penicillium citrinum*, IMI 309573, isolated during a survey of toxigenic fungi from food materials (Trivedi, A.B., Ph.D. thesis, Dr. H.S. Gour University, Sagar, India, 1987). A stock culture has been maintained on potato–dextrose–agar medium. *P. citrinum* was inoculated into broth containing 2% yeast extract and 15% sucrose (YES) and incubated statically at room temperature. Every 5 days, the YES broth was replaced by fresh broth, and citrinin was extracted from the broth with chloroform. Purification was done using a silica gel column. All chloroform fractions were pooled and concentrated under reduced pressure. The resultant residue was applied on silica gel column (4 × 25 cm) equilibrated with benzen-ethylacetate (95 + 5) at room temperature. Elution was carried out with a mixture of benzene and ethyl acetate (95 + 5). Purified citrinin was obtained by recrystallization from ethanol. Purified citrinin gave a single spot on thin-layer chromatography (TLC) (11, 21), a single peak on liquid chromatography (LC) (12), and the same nuclear magnetic resonance (NMR) pattern as that of the standard (13).

Heating of Citrinin and Ochratoxin A

Stock solutions of citrinin (1.18 μg/μL) and ochratoxin A (1.36 μg/μL) were prepared in chloroform and stored at –20°C until use. The exact concentrations of citrinin and ochratoxin A solutions were determined by their absorbances. The molar extinction coefficients of citrinin and ochratoxin A are 1.61×10^4 at 332 nm in chloroform and 4.68×10^3 at 333 nm in ethanol, respectively (25, 26). The stock solution containing 50 μg toxin was pipetted into a 1 mL glass vial and dried under dry nitrogen gas, followed by the addition of 37.5 μL distilled water. To determine the effect of various compounds on the toxicity of citrinin, solutions of each of these compounds were added instead of water. The following compounds were dissolved in distilled water and tested: hydrogen peroxide at a concentration of 0.01% (2.94 mM) to 5% (29.4 mM), glucose (15 mM), fructose (15 mM), D-glucuronic acid (15 mM), D-galacturonic acid (15 mM), monosodium salt of L-glutamic acid (15 mM), glutathione (15 mM), L-cysteine (15 mM), 2-mercaptoethanol (15 mM), dithiothreitol (15 mM), pectin (5 mg/mL), and alginic acid (5 mg/mL). For ochratoxin A, 37.5 μL of various hydrogen peroxide solutions (ranging from 0.05 to 10%), either in 200 mM sodium bicarbonate buffer at various pHs or in 0.1 N NaOH were tested. After the addition of a test solution to the dried toxin powder, the vial was sealed with an air-tight aluminum cap. The vial containing the sample was sonicated 30 s and kept 1 h at room temperature or was placed in an oil bath and heated at a rate of 3°C/min. The vial containing citrinin was heated to 100°C and kept at 100°C for 30 min, while the vial containing ochratoxin A was only heated to 100°C. The sample bottles were then taken out of the oil bath, cooled to room temperature, and lyophilized. All steps were done in UV-free conditions to prevent decomposition or any other change caused by light. Each chemical reaction was done at least in duplicate.

Ultraviolet Spectroscopy

UV absorption spectra were obtained using a UV-Vis spectrophotometer in chloroform; 13 μg/mL citrinin and solutions containing citrinin-related materials were used for assay.

Cytotoxicity Assay

The toxicities of citrinin, citrinin-related materials, ochratoxin A, and each of the toxins that were treated with hydrogen peroxide or with other chemicals (including various saccharides) were assayed using HeLa cells. The complete bioassay has been described elsewhere (11, 27). Briefly, either the toxin or the toxin treated with chemicals was dispersed in fetal bovine serum (FBS) and diluted with Dulbecco's modified Eagle's medium to generate different concentrations; then 50 μL of each test solution was put into 1 well of a 96-well microplate. To each well, 50 μL of the HeLa cell suspension containing 6×10^4 cells/mL was added. Cells in the microwell plate were incubated for 3 days at 37°C in a 5% CO_2 atmosphere. Cell growth was measured by a colorimetric method with the use of MTT, which gave a good correlation

between the cell number and color development upon the reduction of MTT under suitable conditions (27, 28). The color developed was measured with a microplate reader at the test wavelength of 540 nm and the reference wavelength of 620 nm. The absorbance of the control well was that of the well containing the medium and HeLa cells without either the toxin or the toxin treated with chemicals. The percentage of cytotoxicity was calculated using the following equation:

$$\%, \text{cytotoxicity} = (1 - \frac{ab_{tr}}{ab_{co}}) \times 100$$

where ab_{tr} = absorbance of the treated well and ab_{co} = absorbance of the control well.

All experiments were carried out at least in duplicate, and the mean values are shown.

Citrinin or citrinin-related materials were incubated with 0, 0.01, 0.05, and 5% hydrogen peroxide at room temperature for 15, 30, or 60 min, and with 0.05% hydrogen peroxide at 100°C for 30 min, and the samples were diluted 44 times with culture medium. The diluted samples were then added to cell suspensions to yield final concentrations of hydrogen peroxide ranging from 0 to 0.11%, which corresponded to the 0 to 5% hydrogen peroxide used for detoxification.

Results

Citrinin

First, the possible toxic effect of hydrogen peroxide on HeLa cells was examined. Figure 1 shows that the absorbance at 540 nm, which corresponded with the number of cells, increased both with and without hydrogen peroxide, indicating that the cells proliferated well in the presence of hydrogen peroxide. In another experiment, we confirmed that hydrogen peroxide concentrations up to 10% (final concentration in cell suspension of 0.22%) did not negatively affect the proliferation of cells in this system.

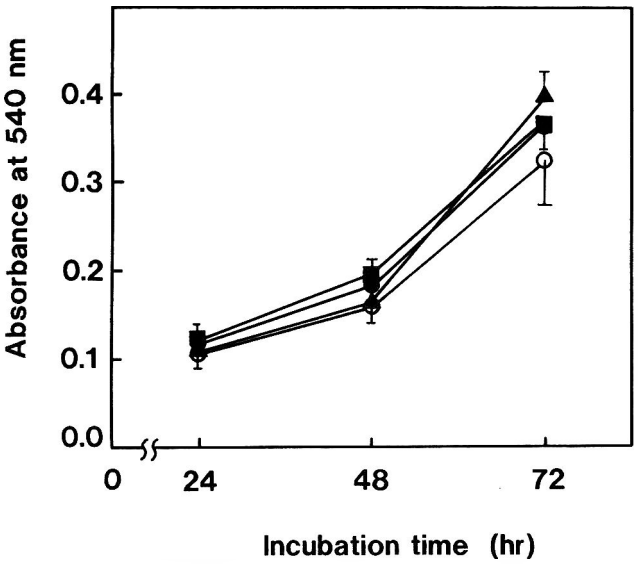


Figure 1. Cytotoxicity of hydrogen peroxide. Absorbance at 540 nm corresponds to the number of HeLa cells, the initial cell number being 3×10^3/100 μL of medium. The hydrogen peroxide concentrations were 0% (○), 0.01% (●), 0.05% (■), and 5% (▲), diluted with culture medium 44 times to yield final concentrations in the cell suspensions, of 0, 0.00023, 0.0011, and 0.113%, respectively.

The effect of hydrogen peroxide on citrinin was investigated next (Figure 2). The citrinin concentration was fixed at 3 μg/well (0.03 μg/μL), which was sufficient to arrest cell proliferation. Citrinin was treated with hydrogen peroxide at concentrations of 0.01% or higher at room temperature, and at a concentration of 0.05% at 100°C, diluted with culture media, and then added to the HeLa cells. The HeLa cells exposed to citrinin treated with hydrogen peroxide concentrations of 0.05% proliferated as well as the control cells without citrinin. This result clearly indicates that the hydrogen peroxide-treated citrinin was not toxic to HeLa cells, i.e., citrinin can be completely detoxified by hydrogen peroxide at room temperature.

The change in the absorption spectrum of citrinin incubated with hydrogen peroxide was measured (Figure 3). The absorption peak at ca 330 nm decreased quickly with time. After 60 min of incubation of citrinin with hydrogen peroxide, completely disappeared. On the other hand, the absorbance at

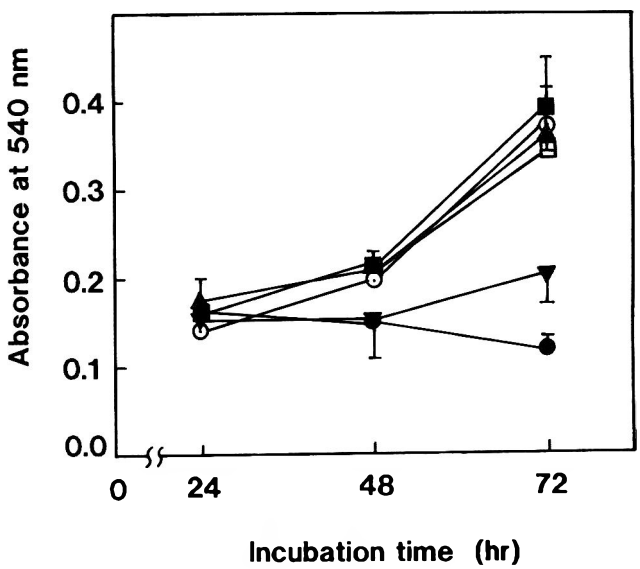


Figure 2. Cytotoxicity of citrinin previously treated with hydrogen peroxide at room temperature. Absorbance at 540 nm corresponds to the number of HeLa cells, the initial cell number being 3×10^3/100 μL of medium. Citrinin was incubated 30 min with hydrogen peroxide at concentrations of 0% (●), 0.01% (▼), 0.05% (■), and 5% (▲) at room temperature, and at a concentration of 0.05% (□) at 100°C. The samples were then diluted with culture medium and added to cell suspensions at a citrinin concentration of 3 μg/100 μL and a hydrogen peroxide concentration resulting from 44-fold dilution. The control experiment with HeLa cells without citrinin and hydrogen peroxide that were similarly incubated in the medium is shown by the open circles (○).

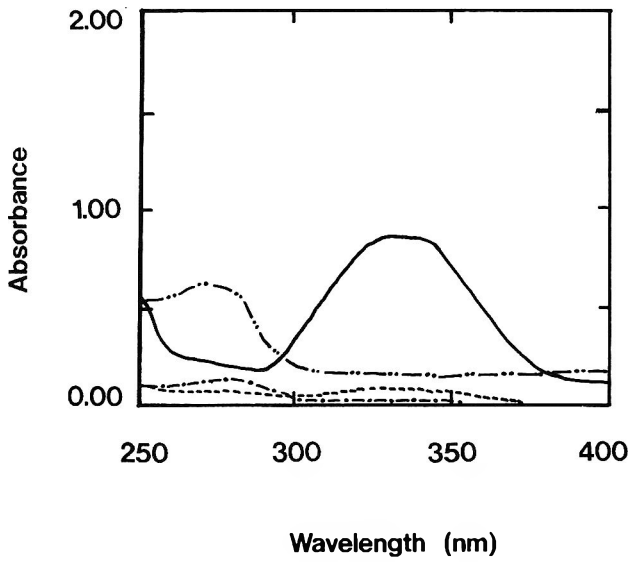


Figure 3. UV spectra in chloroform at 13 μg/mL of citrinin (——) and citrinin incubated with 0.05% hydrogen peroxide for 15 min (– – –), 1 h (– • –), and overnight (– • • –).

278 nm increased with time and gave a relatively high value after overnight incubation of citrinin with hydrogen peroxide, when the absorbance at 330 nm approached zero.

Incubation of citrinin with 0.05% hydrogen peroxide at room temperature appeared to be effective in detoxifying citrinin and was accompanied by the decomposition of the

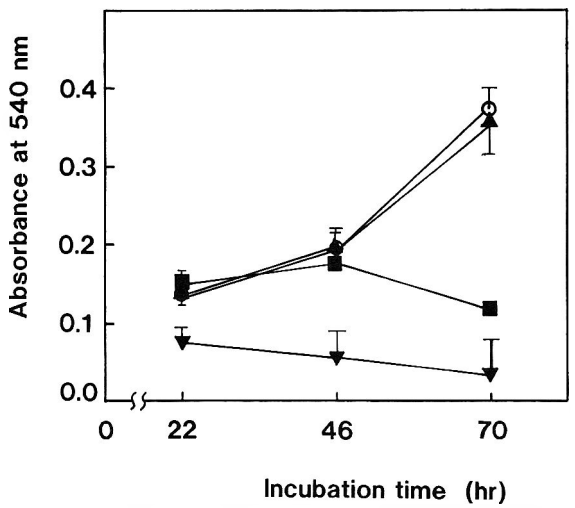
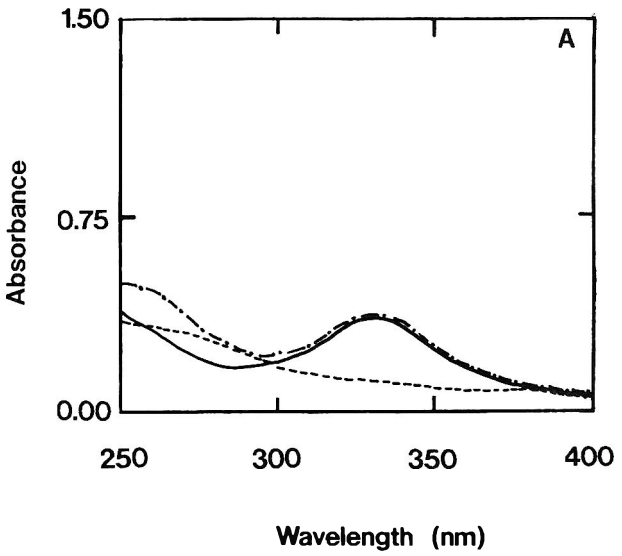


Figure 4. Cytotoxicity of the citrinin heated at 100°C for 30 min under moist conditions (heated citrinin) with hydrogen peroxide. Absorbance at 540 nm corresponds to the number of HeLa cells, the initial cell number being 3×10^3/100 μL of medium. Heated citrinin was incubated with hydrogen peroxide concentrations of 0% (▼), 0.05% at room temperature for 1 h (■), and 0.05% at 100°C for 30 min (▲). The toxin concentration in the cell suspension was 3 μg/100 μL. The control experiment with HeLa cells without either toxin or hydrogen peroxide in the medium is shown by the open circles (○).



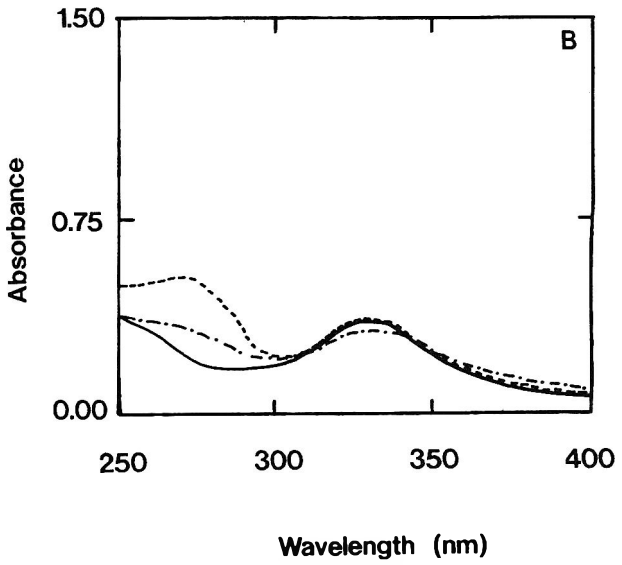


Figure 5. UV spectra of citrinin and heated citrinin treated with hydrogen peroxide or water. Citrinin was heated at 100°C for 30 min under moist conditions (heated citrinin) (———). (A) Heated citrinin was incubated with 0.05% hydrogen peroxide at room temperature for 1 h (—•—), or with 0.05% hydrogen peroxide at 100°C for 30 min (– – – –). (B) Citrinin was heated with water at 100°C for 1 h (— • —) and heated citrinin was again heated with water at 100°C for 30 min (– – – –). Samples were dissolved in chloroform at 13 μg/mL for spectral measurement.

citrinin molecule at room temperature, because a change in the absorption spectra occurred. To determine the effect of heating on citrinin detoxification, the citrinin with hydrogen peroxide was heated at 100°C for 30 min, and its toxicity was examined. Figure 2 showed that such heating had no effect on the toxicity, i.e., the heated sample was also nontoxic.

Table 1. Percent cytotoxicity of ochratoxin A pretreated with hydrogen peroxide at room temperature or while heated at 100°C

Ochratoxin A concn, µg/well	Hydrogen peroxide, %											
	0.0		0.05		0.1		1.0		5.0		10.0	
	RT	100°C	RT	100°C	RT	100°C	RT	100°C	RT	100°C	RT	100°C
3	99	98	96	98	96	98	99	99	99	98	99	99
1	75	81	72	83	68	79	75	78	78	77	63	78
0.5	36	35	32	48	24	36	23	37	44	35	13	34

It is known that citrinin is decomposed to more toxic compound(s) on prolonged heating under moist conditions at 100°C (12). Therefore, an attempt to detoxify such heat-induced toxins from citrinin with hydrogen peroxide was made. First, citrinin was heated under moist conditions at 100°C for 30 min to form the heat-induced product, which is noted as "heated citrinin." This sample was incubated with 0.05% hydrogen peroxide at room temperature, and then its toxicity was tested (Figure 4). The toxicity of heated citrinin, being different from citrinin, was not reduced by incubation with hydrogen peroxide at room temperature, indicating that the toxic compounds, including citrinin H1, in heated citrinin were stable in the presence of hydrogen peroxide at room temperature. However, when heated citrinin was heated to 100°C a second time with 0.05% hydrogen peroxide, its toxicity disappeared. Heating at 100°C for 30 min in the presence of 0.05% hydrogen peroxide was effective and was required to detoxify heated citrinin.

The change in toxicity of heated citrinin upon heating with hydrogen peroxide was identified by a change in its UV absorption spectrum (Figure 5). The spectrum of heated citrinin was not much different from that of citrinin (Figure 3). The absorption maximum was observed at ca 330 nm and was similar to that of citrinin. This spectrum was not changed by incubation with 0.05% hydrogen peroxide for 1 h at room temperature. However, heating it to 100°C with 0.05% hydrogen peroxide for 30 min resulted in an obvious alteration of its spectrum (Figure 3A), while heating with water did not reduce the absorbance at ca 330 nm (Figure 3B). The difference in the UV spectra of heated citrinin and heated citrinin reheated with 0.05% hydrogen peroxide confirms that decomposition of toxic compounds in heated citrinin by the 0.05% hydrogen peroxide solution occurred upon heating.

Effects of Other Chemicals on Citrinin Toxicity

Glucose, fructose, D-glucuronic acid, D-galacturonic acid, L-glutamic acid monosodium salt, pectin, and alginic acid had no effect on the toxicity of citrinin under the conditions tested. 2-Mercaptoethanol and dithiothreitol did reduce the toxicity of citrinin. However, they were not as effective as hydrogen peroxide. In addition, other sulfhydryl reagents did not elicit any effect under the conditions tested.

Ochratoxin A

Ochratoxin A is a more toxic compound than citrinin (29). Cytotoxicities of 3 concentrations of ochratoxin A pre-treated with various concentrations of hydrogen peroxide at room temperature and while heated to 100°C with hydrogen peroxide present were examined (Table 1). At 3, 1, and 0.5 µg/well, no change in the toxicity of ochratoxin A was observed, regardless of whether it was heated or not heated with hydrogen peroxide. In summary, hydrogen peroxide has no effect on the detoxification of ochratoxin A under these conditions. Nevertheless, ochratoxin A can be detoxified by heating it in alkaline conditions (21). However, a strong alkaline solution is required to detoxify it. Therefore, a combination of treatment with a weak alkaline solution, heating, and treatment with hydrogen peroxide was tested with ochratoxin A to determine the conditions for mild detoxification. Table 2 presents the percent cytotoxicity of ochratoxin A after heating it to 100°C with 3 different concentrations of hydrogen peroxide at pHs ranging between 9.2 and 12.5. Below pH 9.8, the addition of hydrogen peroxide gave some decrease in toxicity, i.e., the highest cytotoxicity was observed at 0% hydrogen peroxide. On the contrary, at pHs above 9.8, the addition of hydrogen peroxide diminished the reductions of toxicity due to pH alone; i.e., in this pH range, the addition of hydrogen peroxide was not only ineffective in decreasing the toxicity of ochratoxin A further but instead gave an opposite effect. This finding might be caused by a slight change in pH induced by the addition of hydrogen peroxide, which influenced the toxicity of ochratoxin A in this pH region. Complete detoxification was only achieved with strong alkalinity (pH 10.8–12.5). Some stimulation of cell growth was observed by addition of citrinin treated with a strongly alkaline solution. For each range of pH, the increasing concentration of hydrogen peroxide had no remarkable effect in promoting the detoxification of ochratoxin A.

Discussion

According to Jemmali (30), decontamination of mycotoxin from foodstuffs should destroy, remove, or inactivate the mycotoxin and should not produce any toxic compound. However, decontamination procedures such as heating do not entirely satisfy these criteria for citrinin, because citrinin may change to more toxic compound(s) under a certain heating condition and ochratoxin A cannot be detoxified by heating only.

Table 2. Percent cytotoxicity of ochratoxin A[a] pretreated with varying concentrations of hydrogen peroxide in 0.2 M bicarbonate buffer (of variable pH), heated to 100°C

pH of bicarbonate buffer, 0.2 M	Hydrogen peroxide, %			
	0	0.05	0.5	1.0
9.5–9.6	33	28	36	15
9.7–9.8	29	13	18	19
9.8–10.0	14	18	25	30
10.8[b]–12.5	–23	–15	–13	–16
Without buffer	75	—	—	—

[a] Ochratoxin A concentration; 1 μg/100 μL.
[b] 0.1 N NaOH was added to buffer.

Therefore, some physical and/or chemical treatment in combination with heat treatment is required to obtain effective decontamination of ochratoxin A.

Not only citrinin and ochratoxin A but also most of the other mycotoxins need such a combination for detoxification of food or feed under mild and acceptable conditions.

It was demonstrated that the antimicrobial activity of citrinin disappeared in the presence of cysteine (31), while Ciegler et al. (32) reported that no reaction between citrinin and sulfhydryl reagents was detected. In this study, sulfhydryl reagents were not very effective for detoxifying citrinin, although some effect was observed. Precise investigation is needed to clarify the role of sulfhydryl reagents in detoxification.

Hydrogen peroxide has been used to decontaminate mycotoxins in cereal grains (14, 23). In our experiment, citrinin was detoxified by pretreatment with 0.05% hydrogen peroxide, and heated citrinin was also detoxified upon heating it to 100°C with 0.05% hydrogen peroxide.

The present study reveals hydrogen peroxide detoxifies citrinin, and hydrogen peroxide-treated citrinin does not yield additional toxic compounds after heating. Moreover, the heated citrinin, containing other toxic compounds such as citrinin H1, can be detoxified by reheating it with hydrogen peroxide. Therefore, citrinin-contaminated food and feed should not be heated under moist condition, but instead should be treated with hydrogen peroxide.

Regarding ochratoxin A, we could not observe enough detoxification by treatment with 0.05 to 10% hydrogen peroxide either at room temperature or while heating at 100°C. Detoxification of ochratoxin A can be achieved by heating it to 100°C in pH 10.8–12.5 buffer added with 0.1 N NaOH (21). In the present study, the heating of ochratoxin A in a weakly alkaline buffer was also effective in reducing the toxicity of ochratoxin A. The use of hydrogen peroxide in alkaline solutions seems to be effective in boosting the detoxification of ochratoxin A during heating. However, to obtain complete detoxification, a high pH (above 10) was required. The particular combination of alkaline pH, heating temperature, and percent hydrogen peroxide appeared to be important in determining the optimal method for the complete detoxification of ochratoxin A.

The efficiency of chemical and physical methods for detoxification of mycotoxins can be investigated precisely and over a wide range using this cytotoxicity assay system. The introduction of this assay system might make it possible to evaluate the toxicity of compounds resulting from the decomposition of mycotoxins.

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FOOD CHEMICAL CONTAMINANTS

Solvent-Efficient Thin-Layer Chromatographic Method for the Determination of Aflatoxins B_1, B_2, G_1, and G_2 in Corn and Peanut Products: Collaborative Study

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An interlaboratory study of a solvent-efficient thin-layer chromatographic (TLC) method for the determination of aflatoxins B_1, B_2, G_1, and G_2 was conducted in laboratories located in the United States, France, Tunisia, and Denmark. Eighteen artificially contaminated samples plus blanks of raw peanuts and peanut butter and corn containing varying amounts of aflatoxins B_1, B_2, G_1, and G_2 were distributed to participating laboratories. The method consists of elements of the U.S. Food and Drug Administration (FDA), Contaminants Branch (CB) (AOAC Method 968.22) and FDA, Best Foods (BF) (AOAC Method 970.45) methods with reduced requirements for solvents. Participating laboratories used either visual or densitometric techniques during the final determinative step. Statistical analysis of the data was performed to determine or confirm outliers and to compute repeatability and reproducibility of the method using either visual or densitometric techniques for the determinative step. Reported results from laboratories using a densitometer showed that, for corn, the relative standard deviation for repeatability (RSD_r) for aflatoxin B_1 ranged from 56.6 to 41.7% for contamination levels ranging from 5 to 50 ng/g. For raw peanuts and peanut butter, the RSD_r values for aflatoxin B_1 ranged from 21.3 to 37.3% and 65.9 to 42.1%, respectively, for the contamination levels ranging from 5 to 25 ng/g. RSD_r ranges for aflatoxins B_2, G_1, and G_2 were similar. For reproducibility (R), the RSD_R ranges for aflatoxin B_1 were 41.7–56.6%, 56.6–84.8%, and 26.4–37.3% for corn, peanut butter, and raw peanuts, respectively. Average recoveries for all aflatoxins at all levels were 95.3, 139.0, and 95.6% for corn, peanut butter, and raw peanuts, respectively. When analysts determined aflatoxin concentrations in corn by visual comparison to standards, the RSD_r values for aflatoxin B_1 were 47.8–11.4% for contamination levels ranging from 5 to 50 ng/g. For raw peanuts and peanut butter, the RSD_r values for aflatoxin B_1 were 76.3–12.6% and 33.4–8.8%, respectively, for the contamination levels ranging from 5 to 25 ng/g. RSD_r values for aflatoxins B_2, G_1, and G_2 were similar. The RSD_R values for aflatoxin B_1 were 34.6–90.2%, 45.5–59.3%, and 31.8–78.3% for corn, peanut butter,

and raw peanuts, respectively. Average recoveries for all aflatoxins at all levels were 111.0, 157.6, and 92.3% for corn, peanut butter, and raw peanuts, respectively. High recoveries were noted for aflatoxins in peanut butter determined by either a densitometer or comparison to standards. Generally, increased precision was observed with the method at higher contamination levels. On the basis of the results obtained in this AOAC/IUPAC collaborative study, the solvent-efficient TLC method using densitometry for the quantitative step was adopted first action by AOAC for the determination of aflatoxins B_1, B_2, G_1, and G_2 in corn at levels within the range of 5–50 ng/g, 3–15 ng/g, 10–50 ng/g, and 3–15 ng/g, respectively, by densitometry; for aflatoxins B_1 and B_2 in raw peanuts at levels ranging from 5 to 25 ng/g and from 1.5 to 7.5 ng/g, respectively, by densitometry; and for aflatoxins B_1 and G_1 in corn at 10–50 and 50 ng/g, respectively, and aflatoxins B_1, B_2, and G_1 in raw peanuts at 10–25 ng/g, 7.5 ng/g, and 10–25 ng/g, respectively. Because of high recovery values, additional study is recommended for peanut butter. The solvent-efficient TLC method for determination of aflatoxins B_1, B_2, G_1, and G_2 in corn and peanuts has been adopted first action by AOAC INTERNATIONAL.

Historically, qualitative and quantitative assay procedures for aflatoxins were based on the use of thin-layer (TLC) and liquid (LC) chromatography with authentic samples of the individual, pure mycotoxins used as reference materials (1, 2). These methods, although accurate to low parts-per-billion levels, are time consuming and require extensive sample cleanup, large amounts of toxic solvents, and, occasionally, expensive instrumentation. The U.S. Food and Drug Administration (FDA), Contaminants Branch (CB) TLC method (AOAC Method **968.22**) (3) for the determination of aflatoxins in grains, in particular, is costly and time consuming. Elements of the CB and the FDA, Best Foods (BF) method (AOAC Method **970.45**) (4) were modified and combined, resulting in a method with improved detection and quantitation characteristics as well as a reduction in solvent consumption during column chromatography from 450 to 20 mL/sample (5). These procedures were developed as part of early aflatoxin development programs carried out by FDA, CB and BF. These methods were initially adopted official first action by AOAC in 1968 (CB method) (6) and 1970 (BF method) (7).

For the present solvent-efficient method, aflatoxins are extracted with methanol–water (85 + 15) rather than with methanol–water (55 + 45) as outlined in the BF method. The filtrate is diluted with 10% NaCl solution and defatted with hexane. The toxins are partitioned into chloroform, which is then removed by evaporation, and further purified, as outlined in the CB method, by chromatography on a silica gel column scaled down to use 0.5 g instead of 10 g. The aflatoxins are then quantitated by TLC densitometry or visual comparison to standards. This method was selected for collaborative study under the sponsorship of AOAC and IUPAC.

Collaborative Study

Fourteen laboratories were each furnished with 18 coded test portions and blanks of raw peanuts, peanut butter, and corn artificially contaminated with aflatoxins B_1, B_2, G_1, and G_2. Test portions were prepared by obtaining sufficient quantities of corn, raw peanuts, and peanut butter as free as possible from aflatoxin contamination and adding various amounts of aflatoxins B_1, B_2, G_1, and G_2. Contamination levels ranged from 13 to 65 ng total aflatoxins/g for raw peanuts and peanut butter and from 13 to 130 ng/g for corn. The nonspiked sample served as a negative control.

Collaborators were supplied with 30 silica gel columns (Baker 7086-6), plastic syringe and adaptor, practice test portions, reference standards, method directions, and test portions of each commodity in duplicate at all contamination levels. Participating laboratories could use either densitometry or visual comparison to standards for the final determinative step. The participants were asked to run the practice test portions. If recoveries <75% of labeled value were obtained, they were instructed to contact the originators of the study.

993.17 Aflatoxins in Corn and Peanuts—Thin-Layer Chromatographic Method

First Action 1993

(Applicable to determination of 5–50 ng B_1/g corn, 3–15 ng B_2/g corn, 10–50 ng G_1/g corn, 3–15 ng G_2/g corn, 5–25 ng B_1/g raw peanuts and 1.5–7.5 ng B_2/g raw peanuts by densitometry; 10–50 ng B_1/g corn, 50 ng G_1/g corn, 10–25 ng B_1/g raw peanuts, 7.5 ng B_2/g raw peanuts, and 10–25 ng G_1/g raw peanuts by visual comparison)

Method Performance:

See Tables **993.17A** and **993.17B** for method performance data.

(*Caution*: Aflatoxins are extremely potent carcinogens to many animals. Neither effects of aflatoxins on humans nor possible routes of entry are presently known. Observe precautions given in introductory statement, Chapter 49, Natural Poisons.

See Appendix: Laboratory Safety for precautions in using organic solvents.

Submitted for publication February 15, 1993.

The recommendation was approved by the Committee on Natural Toxins and was adopted by the Official Methods Board of the Association. See "Changes in Official Methods of Analysis" (1994) *J. AOAC Int.* **77**, Jan/Feb issue, and "Official Methods Board Actions" (1993) *The Referee*, **17**, July issue.

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Note: This study has been carried out under the sponsorship of AOAC INTERNATIONAL and the IUPAC Commission on Food Chemistry.

Table 993.17A. Method performance for determination of aflatoxins B_1, B_2, G_1, and G_2 in corn and peanuts by thin-layer chromatographic method with densitometer estimation

Product	Aflatoxin	Spike, ng/g	Recovery mean, %	s_r	s_R	RSD_r, %	RSD_R, %
Corn	B_1	5	88	1.98	1.98	56.6	56.6
		10	83	2.58	2.58	41.7	41.7
		50	76	14.28	14.25	47.5	47.3
	B_2	3	94	0.64	1.18	26.8	49.3
		15	118	5.37	6.32	45.5	53.6
	G_1	10	102	3.31	4.61	43.6	60.7
		50	91	13.07	15.42	36.1	42.6
	G_2	3	90	1.12	1.44	48.8	62.8
		15	104	4.68	4.68	45.0	45.0
Raw peanuts	B_1	5	78	0.66	0.82	21.3	26.4
		10	83	2.31	2.31	37.3	37.3
		25	84	4.36	4.83	26.1	28.9
	B_2	1.5	103	0.38	0.50	38.1	50.3
		3	74	0.67	0.67	37.3	37.3
		7.5	123	2.31	2.31	37.2	37.2

Grinding of dry samples may result in airborne dust. Even if no toxin is present, there is potential harm from inhalations of mold spores or from allergic response to inhaled dust. Use protective mask and/or dust collector. Prepare samples in area separate from analytical laboratory.

Note: Soak all used laboratory ware and pipet tips in 10% solution of household bleach (ca 5.25% NaOCl) before discarding. To clean reusable containers between samples, immerse whole container in 10% household bleach 15 min and drain. Immerse in water 15 min and drain, 2×. Let container drip dry.)

A. Principle

Aflatoxins are extracted from samples with methanol-water. Filtrate is diluted with NaCl solution and defatted with hexane. Aflatoxins are partitioned into chloroform which is then removed by evaporation. Aflatoxins are purified by chromatography on 0.5 g silica gel column, and quantitated by thin-layer chromatography (TLC) on silica gel 60 plate with densitometry or visual estimation.

B. Apparatus

(**a**) *Wrist-action shaker.*—Capable of holding 4–8 250 mL flasks (Burrell Corp., Pittsburgh, PA, is suitable source).

(**b**) *Silica gel column.*—6 mL disposable column, packed with 40 μm (60Å) silica gel.

(**c**) *Vacuum apparatus.*—Equipped with vacuum gauge/flow controller and manifold fitted with 10 female Luer connectors (Baker extraction system, J.T. Baker Chemical Co., is suitable).

(**d**) *Vials.*—2 dram (8 mL), with foil or Teflon-lined screw caps.

(**e**) *TLC plate.*—20 × 20 cm glass plate coated with 0.25 mm thick silica gel without fluorescent indicator (precoated, silica gel 60 plates, E. Merck, Gibbstown, NJ, are suitable).

Table 993.17B. Method performance for determination of aflatoxins B_1, B_2, G_1, and G_2 in corn and peanuts by thin-layer chromatographic method with visual estimation

Product	Aflatoxin	Spike, ng/g	Recovery mean, %	s_r	s_R	RSD_r, %	RSD_R, %
Corn	B_1	10	100	0.86	4.25	11.4	56.7
		50	92	12.66	12.66	34.6	34.6
	G_1	50	96	11.67	11.67	30.3	30.3
Raw peanuts	B_1	10	101	0.96	2.71	12.6	35.7
		25	87	5.53	5.53	31.8	31.8
	B_2	7.5	120	1.67	2.69	27.8	44.9
	G_1	10	69	0.63	1.77	12.1	34.0
		25	76	6.52	6.54	42.9	43.0

(**f**) *Viewing cabinet.*—270 × 270 mm base minimum, equipped with 15 W long-wave ultraviolet (UV) lamp (Chromato-Vue, Ultra-Violet Products, Inc., San Gabriel, CA, is suitable).

(**g**) *Fluorodensitometer (TLC scanner).*—Capable of scanning in reflectance mode by fluorescence, equipped with high pressure Hg lamp, monochrometer for adjustment to excitation 366 nm, and emission cut-off filter 420 nm (CAMAG TLC Scanner, Applied Analytical Industries, Wilmington, NC, is suitable).

C. Reagents

(**a**) *Solvents.*—Methanol, hexane, chloroform, anhydrous ethyl ether (100%), dichloromethane, acetone, and isopropanol.

(**b**) *Aflatoxins standard solution.*—Prepare in benzene–acetonitrile (98 + 2) as in **971.22** to contain 0.5 μg/mL each B_1 and G_1 and 0.15 μg/mL each B_2 and G_2.

D. Extraction and Partition

Weigh 50 g (ground to pass 20 mesh) corn or peanuts into 500 mL glass-stoppered Erlenmeyer flask. Add 200 mL methanol–H_2O (85 + 15) and secure stopper with masking tape. Shake vigorously by hand until samples show no clumps. Shake 30 min on wrist-action shaker, *B*(**a**), and filter mixture through medium fluted paper. Collect 40 mL filtrate in 50 mL graduated cylinder. Transfer filtrate to 125 mL separatory funnel. Add 40 mL 10% NaCl solution, mix, and add 25 mL hexane. Shake 1 min. Let phases separate, drain lower (aqueous) phase into second 125 mL separatory funnel, and discard upper phase.

Extract aflatoxins from aqueous phase with two 25 mL portions $CHCl_3$; shake 1 min each time. Combine $CHCl_3$ fractions in 125 mL Erlenmeyer flask and evaporate to dryness on steam bath.

E. Silica Gel Column Chromatography

Attach silica gel column, *B*(**b**), to extraction system, *B*(**c**), (or clamp to stand if using gravity flow only). Condition column by washing with 3 mL hexane, then 3 mL dichloromethane using vacuum (flow rate 6 mL/min), or let drip freely unassisted by suction.

Check column suitability by adding aflatoxin B_1 standard (3 mL dichloromethane containing 100 ng aflatoxin B_1) to 0.5 g silica gel column. Recovery must be ≥90% by this method.

Dissolve sample residue, from *D*, in 3 mL dichloromethane and add to column. Let sample drip freely (flow rate ca 3 mL/min, apply vacuum if needed). Rinse sample residue container with two 1 mL portions of dichloromethane and add rinses to column. Wash column with 3 mL hexane, 3 mL anhydrous ethyl ether, and then 3 mL dichloromethane. (Use vacuum, flow rate 6 mL/min, or use syringe and adapter to apply pressure to increase solvent flow if necessary. Do not pull up syringe plunger while it is still attached to column.) Turn off vacuum, remove extraction system cover, and place vial, *B*(**d**), under each column (test tube rack can be used to hold vials).

Elute aflatoxins (without vacuum) with two to four 3 mL portions (according to results of column suitability test) of $CHCl_3$–acetone (9 + 1). Evaporate eluate to dryness on steam bath under stream of nitrogen.

F. Thin-Layer Chromatography–Fluorodensitometry Determination

Dissolve sample extract from *E* in 250 μL $CHCl_3$. Spot plate, *B*(**e**), with 5 μL $CHCl_3$ sample solution in duplicate and 2, 5, 10, and 20 μL aflatoxin standard solution, *C*(**b**). Randomize standard and sample spots across plate so duplicate sample spots are not next to each other and standard spots are dispersed evenly. To avoid errors, prepare spotting plan, either on plate or in notebook, prior to spotting.

Develop plate 1 h with $CHCl_3$–acetone (9 + 1). Evaporate solvent 5 min in fume hood followed by 2 min in 50° forced draft oven.

Examine plate under long-wave UV light to determine presence or absence of aflatoxins.

Quantitate by fluorodensitometric measurement. Scan sample and aflatoxin reference spots (transmission or reflectance mode, excitation 365 nm and emission cutoff 430 nm). At end of plate scan, rescan 1st or 2nd lane. Scans of same spots should be within ±5%; if not, rescan entire plate.

G. Calculation

Calculate concentration of aflatoxin B_1 in sample, using following formula:

$$B_1,\ \text{ng/g} = \frac{250 \times R_u}{5 \times R_s \times 10}$$

where 250 = μL sample extract volume; R_u = average densitometer response for B_1 spots of sample extract duplicates; 5 = μL sample extract spotted; R_s = calculated average densitometer response/ng for 4 B_1 standard spots; 10 = g sample represented by extract.

Calculate concentrations of aflatoxins B_2, G_1, and G_2 similarly.

H. Thin-Layer Chromatography–Visual Estimation

Dissolve sample extract from *E* in 250 μL $CHCl_3$ and proceed as in **968.22F(a)–(d)**.

Ref.: JAOAC **77**, May/June issue (1994).

Results and Discussion

Results of analyses reported by each participating laboratory are presented in Table 1 for those using a densitometer or visual comparison of standards. Early in the study some laboratories had difficulty eluting the aflatoxin standards from the silica gel column with the practice samples. This difficulty was corrected by increasing the elution volume from 6 mL as specified in the method. Therefore, collaborators were asked to spike a test portion of extract and use 3 mL increments of eluting solvent for better recovery and use that volume for the coded samples. Participating laboratories ran the coded sam-

Table 1. Aflatoxin concentrations (ng/g) in artificially contaminated corn and peanut products by densitometry and visual comparison to standards using a solvent-efficient TLC procedure: Collaborative study results. Collaborators 1 through 8 used densitometric determination. Others used visual determination

Lab.	Aflatoxin added	Spiking level in corn, ng/g[a]							Spiking level in peanut butter, ng/g[a]							Spiking level in raw peanuts, ng/g[a]						
		0	13	13	26	26	130	130	0	13	13	26	26	65	65	0	13	13	26	26	65	65
1	B_1	0.0	3.3	3.8	7.9	5.0	33.2	38.5	2.5	7.7	2.2	2.3	9.5	26.5	24.8	0.0	2.9	3.8	5.7	6.0	16.5	14.9
	B_2	0.0	1.2	1.2	2.6	3.0	10.4	11.9	0.0	1.8	0.6	1.0	2.8	7.0	6.9	0.0	1.0	1.3	1.6	1.7	9.4	5.1
	G_1	0.0	3.2	3.3	7.7	4.3	41.2	41.6	0.8	6.7	5.5	9.1	11.9	37.2	33.9	0.0	3.2	3.2	5.9	1.9	13.7	11.1
	G_2	0.0	1.0	0.9	2.3	1.0	10.6	12.1	0.0	2.0	1.7	3.2	4.2	11.0	8.9	0.0	0.8	0.8	1.8	1.0	2.7	3.8
	Total	0.0	8.6	9.1	20.5	13.3	95.4	104.1	3.3	18.2	9.9	15.5	28.4	81.8	74.6	0.0	7.9	8.9	14.9	10.5	42.3	34.8
2	B_1	0.0	3.6	5.4	7.7	6.0	35.3	34.4	2.3	7.4	6.1	13.2	12.2	21.3	27.2	0.0	4.0	3.7	7.1	6.9	17.4	24.5
	B_2	0.0	0.7	1.2	1.7	1.1	9.9	8.7	0.0	1.5	2.0	4.0	3.0	4.8	7.0	0.0	2.1	1.3	2.5	2.7	6.0	8.6
	G_1	0.0	5.3	5.2	7.7	5.3	43.1	43.7	0.0	2.9	5.6	7.6	6.6	18.5	24.2	0.0	6.7	3.7	9.3	7.8	16.1	35.2
	G_2	0.0	0.0	0.0	2.6	1.6	13.0	12.0	0.0	2.1	5.5	3.2	2.5	6.0	11.2	0.0	2.3	2.8	4.7	4.3	7.2	11.3
	Total	0.0	9.6	11.8	19.7	14.0	101.3	98.8	2.3	13.9	19.2	28.0	24.3	50.6	69.6	0.0	15.1	11.5	23.6	21.7	46.7	79.6
3	B_1	0.0	3.7	3.6	6.0	3.9	25.5	38.3	0.0	3.9	5.0	5.9	6.2	8.8	9.0	0.0	2.3	3.0	6.0	7.2	12.1	0.0
	B_2	0.0	1.1	1.3	2.1	1.5	9.5	13.4	0.0	1.3	1.2	1.6	2.0	2.9	2.6	0.0	0.9	1.1	2.3	2.3	4.3	0.0
	G_1	0.0	3.4	3.2	5.3	3.0	23.4	37.8	0.0	3.0	4.2	4.0	5.8	8.4	7.5	0.0	2.1	2.3	6.0	5.5	8.3	0.0
	G_2	0.0	0.9	1.2	1.6	1.5	7.4	11.8	0.0	1.3	1.3	1.6	1.8	2.6	2.0	0.0	0.8	0.7	2.6	2.2	3.5	0.0
	Total	0.0	9.1	9.3	15.0	9.9	65.8	101.3	0.0	9.5	11.7	13.1	15.8	22.7	21.1	0.0	6.1	7.1	16.9	17.2	28.2	0.0
4	B_1	0.0	5.3	0.0	5.4	8.8	41.0	26.1	1.5	0.4	3.9	2.2	6.7	11.1	11.6	0.0	2.0	4.2	2.6	6.3	16.6	12.0
	B_2	0.0	2.3	0.0	4.1	4.1	21.0	11.7	0.9	0.1	0.8	0.3	2.4	4.7	3.7	0.0	0.7	1.7	0.9	2.7	7.5	6.1
	G_1	0.0	8.2	0.0	7.6	10.9	62.7	52.2	1.4	0.4	2.4	2.5	7.9	14.2	13.5	0.0	2.1	5.4	2.0	7.1	24.1	12.8
	G_2	0.0	1.6	0.0	1.5	1.2	9.7	15.0	1.5	0.2	0.9	0.0	0.9	5.1	3.9	0.0	0.7	1.2	0.7	1.8	5.9	5.2
	Total	0.0	17.9	0.0	18.6	25.0	134.4	105.0	5.3	1.1	8.0	5.5	17.9	35.1	32.7	0.0	5.5	12.5	6.2	17.9	54.1	36.1
5	B_1	0.0	5.1	0.0	8.6	3.7	91.9	156.5	3.4	33.1	12.3	12.5	0.0	23.7	42.8	0.0	2.0	1.8	6.8	11.6	15.9	11.2
	B_2	0.0	1.6	0.0	2.5	1.4	25.3	18.7	0.0	3.2	2.5	4.1	0.0	6.5	11.1	0.0	0.6	0.0	2.9	1.7	8.4	5.2
	G_1	0.0	6.2	0.0	13.3	5.1	86.7	190.0	2.1	16.6	8.2	24.9	0.0	27.0	34.2	0.0	5.2	0.0	21.7	8.2	55.1	6.0
	G_2	0.0	2.0	0.0	3.9	2.0	25.9	33.0	0.7	3.7	2.0	4.5	0.0	8.4	10.7	0.0	1.4	0.0	3.8	2.8	10.4	2.7
	Total	0.0	15.0	0.0	28.4	12.1	229.8	398.2	6.2	56.6	25.0	46.0	0.0	65.7	98.9	0.0	9.1	1.8	35.1	24.3	89.8	25.1
6	B_1	0.0	3.1	3.3	6.9	2.2	9.9	39.0	2.5	9.4	9.4	14.1	16.1	32.7	42.0	0.0	3.9	3.9	4.0	8.0	25.6	20.3
	B_2	0.0	1.1	1.1	2.3	1.0	4.1	12.7	0.4	1.4	1.4	2.3	3.1	6.0	6.9	0.0	1.2	1.3	1.3	2.3	7.7	6.1
	G_1	0.0	3.2	3.6	6.8	2.4	8.8	42.0	0.0	5.5	5.9	9.3	9.8	27.7	47.6	0.0	3.0	3.6	3.2	5.5	24.1	18.8
	G_2	0.0	1.1	1.2	2.3	1.0	3.6	13.8	0.0	1.3	1.7	2.4	3.1	6.4	8.9	0.0	1.1	1.6	1.3	2.1	7.9	6.3
	Total	0.0	8.5	9.2	18.3	6.6	26.4	107.5	2.9	17.6	18.4	28.1	32.1	72.8	105.4	0.0	9.2	10.4	9.8	17.9	65.3	51.5
7	B_1	0.0	4.0	6.0	13.0	13.0	44.0	4.0	1.0	8.2	17.0	16.5	5.0	13.0	30.0	0.0	3.5	3.8	—[b]	4.4	12.0	25.0
	B_2	0.0	1.5	2.0	4.5	3.0	17.5	2.0	0.0	3.2	4.5	1.4	2.4	5.5	12.0	0.0	7.5	1.4	—	1.4	3.5	10.0
	G_1	0.0	3.8	5.0	12.5	20.0	44.0	12.0	0.0	6.5	12.0	21.0	5.5	18.0	38.0	0.0	14.5	5.7	—	9.5	13.0	35.0
	G_2	0.0	0.7	1.5	3.4	6.5	17.4	5.0	0.0	2.8	2.7	5.8	1.3	6.3	15.0	0.0	2.8	1.9	—	3.0	5.0	10.0
	Total	0.0	10.0	14.5	33.4	42.5	122.9	23.0	1.0	20.7	36.2	44.7	14.2	42.8	95.0	0.0	28.3	12.8	—	18.3	33.5	80.0

Table 1. ***(continued)***

Lab.	Aflatoxin added	Spiking level in corn, ng/g[a]							Spiking level in peanut butter, ng/g[a]							Spiking level in raw peanuts, ng/g[a]						
		0	13	13	26	26	130	130	0	13	13	26	26	65	65	0	13	13	26	26	65	65
8	B1	0.0	4.0	2.5	9.7	5.2	26.4	27.4	5.7	12.0	7.6	9.0	13.5	23.8	0.0	0.0	3.3	2.3	8.3	3.8	13.5	16.6
	B2	0.0	0.9	0.6	1.5	1.3	6.3	6.0	1.1	1.7	1.5	1.7	1.9	4.0	0.0	0.0	0.8	0.6	1.2	1.0	3.0	2.6
	G1	0.0	3.5	2.3	5.1	4.9	25.5	26.4	2.6	6.2	6.5	5.2	6.4	15.7	0.0	0.0	2.2	1.8	5.2	3.1	12.1	8.5
	G2	0.0	1.1	0.6	2.6	1.3	7.5	7.3	2.4	2.5	2.6	2.3	2.2	5.6	0.0	0.0	0.8	0.7	2.1	4.3	4.1	2.4
	Total	0.0	9.5	6.1	18.9	12.7	65.7	67.0	11.8	22.4	18.2	18.1	24.0	49.2	0.0	0.0	7.1	5.4	10.4	12.2	32.7	30.1
9	B1	0.0	8.0	9.0	12.0	12.0	52.0	38.0	0.0	10.0	10.0	20.0	19.0	26.0	30.0	3.0	6.0	0.0	12.0	12.0	20.0	20.0
	B2	0.0	4.0	4.0	8.0	8.0	18.0	22.0	0.0	7.0	3.0	14.0	8.0	8.0	14.0	1.0	4.0	0.0	6.0	8.0	10.0	11.0
	G1	0.0	8.0	9.0	19.0	10.0	52.0	38.0	0.0	10.0	9.0	18.0	15.0	26.0	25.0	3.0	6.0	0.0	10.0	18.0	18.0	20.0
	G2	0.0	4.0	6.0	9.0	8.0	40.0	22.0	8.0	8.0	8.0	14.0	10.0	14.0	14.0	1.0	4.0	0.0	8.0	11.0	14.0	11.0
	Total	0.0	24.0	28.0	48.0	38.0	162.0	120.0	8.0	35.0	30.0	66.0	52.0	74.0	83.0	8.0	20.0	0.0	36.0	49.0	62.0	62.0
10	B1	0.0	3.8	3.8	4.7	8.8	40.6	40.6	1.9	6.7	8.8	12.5	12.5	23.2	23.2	0.0	3.8	3.1	6.7	7.8	16.3	23.2
	B2	0.0	1.2	1.2	2.0	2.6	12.2	12.2	0.0	2.0	2.6	3.8	3.8	7.0	7.0	0.0	1.2	0.9	2.0	2.3	4.9	7.0
	G1	0.0	3.8	3.8	6.7	8.8	40.6	40.6	0.0	5.5	8.8	8.8	9.6	23.2	23.2	0.0	3.8	3.1	6.7	5.5	16.3	23.2
	G2	0.0	1.2	1.2	2.0	2.6	12.2	12.2	0.0	1.6	2.6	2.6	2.9	7.0	7.0	0.0	1.2	0.9	2.0	1.6	4.9	7.0
	Total	0.0	10.0	10.0	17.4	22.8	105.6	105.6	1.9	15.8	22.8	27.7	28.8	60.4	60.4	0.0	10.0	8.0	17.4	17.2	42.4	60.4
11	B1	0.0	3.0	0.0	6.0	6.0	36.0	19.0	0.0	6.0	5.0	10.0	11.0	25.0	22.0	0.0	0.0	0.0	5.0	5.0	17.0	14.0
	B2	0.0	0.0	0.0	2.0	2.0	12.0	12.0	0.0	2.0	2.0	3.0	3.0	8.0	8.0	0.0	0.0	0.0	1.0	2.0	6.0	5.0
	G1	0.0	3.0	0.0	6.0	5.0	33.0	38.0	0.0	5.0	5.0	9.0	10.0	25.0	29.0	0.0	0.0	0.0	4.0	4.0	15.0	10.0
	G2	0.0	0.0	0.0	0.0	0.0	6.0	6.0	0.0	0.0	0.0	2.0	2.0	8.0	6.0	0.0	0.0	0.0	0.0	0.0	1.0	0.0
	Total	0.0	6.0	0.0	14.0	13.0	87.0	75.0	0.0	13.0	12.0	24.0	26.0	66.0	65.0	0.0	0.0	0.0	10.0	11.0	39.0	29.0
12	B1	0.0	5.0	0.0	8.0	10.0	42.0	43.0	9.0	11.0	4.0	14.0	17.0	27.0	21.0	0.0	4.0	3.0	4.0	6.0	12.0	11.0
	B2	0.0	1.0	0.0	2.0	2.0	11.0	11.0	0.0	3.0	1.0	3.0	5.0	7.0	5.0	0.0	1.0	1.0	1.0	1.0	3.0	3.0
	G1	0.0	5.0	0.0	9.0	11.0	50.0	50.0	0.0	12.0	5.0	12.0	16.0	31.0	25.0	0.0	4.0	4.0	5.0	6.0	12.0	5.0
	G2	0.0	1.0	0.0	2.0	3.0	12.0	12.0	0.0	10.0	1.0	3.0	5.0	11.0	6.0	0.0	1.0	1.0	1.0	2.0	4.0	1.0
	Total	0.0	12.0	0.0	21.0	26.0	115.0	116.0	9.0	36.0	11.0	32.0	43.0	76.0	57.0	0.0	10.0	9.0	11.0	15.0	31.0	20.0
13	B1	0.0	5.0	6.3	10.6	10.0	35.0	30.0	1.3	8.3	10.6	12.5	11.2	21.0	20.0	0.0	5.0	2.5	7.6	10.0	12.6	25.0
	B2	0.0	1.0	2.0	4.4	4.0	16.0	16.0	0.0	2.5	4.0	5.0	3.8	8.0	7.5	0.0	1.2	0.8	2.5	3.6	3.8	7.5
	G1	0.0	5.0	6.3	5.0	10.0	30.0	27.5	0.0	8.8	3.2	6.9	8.8	15.0	20.0	0.0	5.0	2.5	2.5	3.8	5.0	20.0
	G2	0.0	1.0	2.0	2.0	2.5	15.0	3.0	0.0	2.5	1.2	3.6	2.5	5.0	4.0	0.0	0.6	0.8	1.3	1.3	2.0	5.0
	Total	0.0	12.0	16.5	22.0	26.5	96.0	76.5	1.3	22.0	19.0	28.0	26.2	49.0	51.5	0.0	11.8	6.6	13.9	18.6	23.4	57.5
14	B1	0.0	0.0	0.0	0.0	0.0	12.5	50.0	0.0	0.0	0.0	0.0	0.0	0.0	5.0	0.0	25.0	0.0	7.5	7.5	25.0	12.5
	B2	0.0	0.0	0.0	0.0	0.0	3.8	15.0	0.0	0.0	0.0	0.0	0.0	0.0	3.0	0.0	7.5	0.0	3.0	3.0	7.5	3.8
	G1	0.0	0.0	0.0	0.0	0.0	12.5	50.0	0.0	0.0	0.0	0.0	0.0	0.0	5.0	0.0	25.0	0.0	7.5	7.5	25.0	12.5
	G2	0.0	0.0	0.0	0.0	0.0	3.8	15.0	0.0	0.0	0.0	0.0	0.0	0.0	3.0	0.0	7.5	0.0	3.0	3.0	7.5	3.8
	Total	0.0	0.0	0.0	0.0	0.0	32.6	130.0	0.0	0.0	0.0	0.0	0.0	0.0	16.0	0.0	65.0	0.0	21.0	21.0	65.0	32.6

[a] Spiked/calculated concentrations, ng/g; Duplicate samples provided at each concentration except nonspiked sample.

Table 2. Statistical analysis of laboratory data for solvent-efficient TLC method for the determination of aflatoxins B_1, B_2, G_1, and G_2 by densitometric determination

No. of labs	Level, ng/g	Analyte	Av. recovery, %[a]	r	R	RSD_r, %	RSD_R, %	Outliers
Corn								
8	5	B_1	88	1.98	1.98	56.6	56.6	0
8	1.5	B_2	110	0.80	0.80	72.8	72.8	0
8	5	G_1	93	2.60	2.60	70.2	70.2	0
8	1.5	G_2	86	0.72	0.72	80.1	80.1	0
7[b]	1.5	G_2	98	0.75	0.75	74.9	74.9	1
	13	Total[b]	97					
8	10	B_1	94	2.44	3.13	34.1	44.1	0
7[b]	10	B_1	83	2.58	2.58	41.7	41.7	1
8	3	B_2	94	0.64	1.18	26.8	49.3	0
8	10	G_1	102	3.31	4.61	43.6	60.7	0
8	3	G_2	90	1.12	1.44	48.8	62.8	0
	26	Total[b]	92					
8	50	B_1	107	21.00	26.71	50.0	87.4	0
7[b]	50	B_1	76	14.28	14.25	47.5	47.3	1
8	15	B_2	118	5.37	6.32	45.5	53.6	0
8	50	G_1	123	28.57	42.92	58.3	87.6	0
7[b]	50	G_1	91	13.07	15.42	36.1	42.6	1
8	15	G_2	128	4.74	7.71	37.0	60.2	0
7[b]	15	G_2	104	4.68	4.68	45.0	45.0	1
	130	Total[b]	97					
		Overall[c]	95.3					
Peanut butter								
8	5	B_1	225	6.00	7.72	65.9	84.8	0
8	1.5	B_2	179	0.53	1.14	29.6	63.2	0
8	5	G_1	153	2.67	3.89	43.7	63.7	0
8	1.5	G_2	214	0.95	1.22	45.4	58.0	0
	13	Total	193					
8	10	B_1	120	4.88	5.18	54.2	57.5	0
8	3	B_2	88	1.35	1.35	61.3	61.3	0
8	10	G_1	115	7.52	7.53	87.4	87.4	0
8	3	G_2	96	1.62	1.62	67.5	67.5	0
	26	Total	105					
8	25	B_1	109	9.18	12.34	42.1	56.6	0
8	7.5	B_2	114	2.31	3.02	40.5	53.0	0
8	25	G_1	114	8.41	13.32	36.9	58.4	0
8	7.5	G_2	140	3.09	3.98	44.1	56.9	0
	65	Total	119					
		Overall	139.0					
Raw peanuts								
8	5	B_1	78	0.66	0.82	21.3	26.4	0
8	1.5	B_2	146	1.61	1.73	107.1	115.3	0
7[b]	1.5	B_2	103	0.38	0.50	38.1	50.3	0
8	5	G_1	100	2.76	3.26	68.9	81.6	0
7[b]	5	G_1	79	1.84	1.84	57.6	57.6	1
8	1.5	G_2	127	0.47	0.85	36.4	65.1	0
	13	Total[b]	97					

Table 2. *(continued)*

No. of labs	Level, ng/g	Analyte	Av. recovery, %[a]	r	R	RSD_r, %	RSD_R, %	Outliers
Raw peanuts *(continued)*								
8	10	B_1	83	2.31	2.31	37.3	37.3	0
8	3	B_2	74	0.67	0.67	37.3	37.3	0
8	10	G_1	86	4.52	4.98	69.5	76.6	0
8	3	G_2	102	0.80	1.27	30.8	48.7	0
	26	Total	86					
8	25	B_1	84	4.36	4.83	26.1	28.9	0
8	7.5	B_2	123	2.31	2.31	37.2	37.2	0
8	25	G_1	97	14.73	14.73	76.3	76.3	0
8	7.5	G_2	112	3.27	3.27	58.4	58.4	0
	65	Total	104					
		Overall	95.6					

[a] Average natural aflatoxin B_1 contamination levels ("0" columns for all commodities, Table 1) found by collaborators for corn, peanut butter, and raw peanuts were 0.0, 2.2, and 0.21 ng/g, respectively; calculated recovery values do not take into account this source of aflatoxins.
[b] Outlier(s) removed.
[c] Overall average recovery values include outliers.

ples by using 6–12 mL to elute aflatoxins off the column. Not all collaborators received instructions in time, but several had already increased the solvent elution volume on their own. This problem occurred because the manufacturer of the columns changed the production program immediately before initiation of the collaboratory study. Experiments in the authors' laboratory prior to distribution of the samples and supplies showed that 6 mL was sufficient elution volume. The changing of the product specifications by the manufacturer (unknown to the analyst) highlights the importance of confirming the performance characteristics of the columns before conducting any analysis. A column testing section has been added to the method.

For most participating laboratories, emulsions were not a problem and filtering was relatively quick. The method provides very clean extracts. Two laboratories, however, did encounter emulsions during the hexane extraction. One laboratory resolved the problem by centrifuging and transferring the

Table 3. Statistical analysis of laboratory data for solvent-efficient TLC method for the determination of aflatoxins B_1, B_2, G_1, and G_2 by visual determination

No. of labs	Level, ng/g	Analyte	Av. recovery, %[a]	r	R	RSD_r, %	RSD_R, %	Outliers
Corn								
6	5	B_1	91	1.72	3.25	47.8	90.2	0
6	1.5	B_2	120	0.41	1.51	34.0	127.6	0
6	5	G_1	91	1.72	3.25	47.8	90.2	0
6	1.5	G_2	136	0.72	2.00	51.7	142.6	0
	13	Total	110					
6	10	B_1	100	0.86	4.25	11.4	56.7	0
6	3	B_2	123	0.21	2.77	6.8	89.4	0
6	10	G_1	101	3.08	5.26	41.1	70.1	0
6	3	G_2	104	0.47	3.14	18.0	120.9	0
	26	Total	107					
6	50	B_1	92	12.66	12.66	34.6	34.6	0
6	15	B_2	134	3.43	4.57	25.6	34.1	0
5[b]	15	B_2	142	1.26	3.53	8.9	27.0	1
6	50	G_1	96	11.67	11.67	30.3	30.3	0
6	15	G_2	132	7.05	10.29	53.0	77.4	0
	130	Total[b]	116					
		Overall[c]	111.0					

Table 3. ***(continued)***

No. of labs	Level, ng/g	Analyte	Av. recovery, %[a]	r	R	RSD_r, %	RSD_R, %	Outliers
Peanut butter								
6	5	B_1	167	2.24	3.97	33.4	59.3	0
6	1.5	B_2	243	1.36	1.89	56.6	78.8	0
6	5	G_1	150	2.75	3.88	45.9	64.7	0
6	1.5	G_2	290	2.63	3.70	90.8	127.5	0
5[b]	1.5	G_2	239	0.52	3.32	21.7	138.5	1
	13	Total[b]	200					
6	10	B_1	155	1.02	6.57	8.8	56.6	0
6	3	B_2	172	1.88	3.88	42.7	88.2	0
6	10	G_1	127	1.59	5.80	16.7	61.1	0
6	3	G_2	156	1.34	4.30	33.6	107.5	0
	26	Total	153					
6	25	B_1	101	2.70	9.24	13.3	45.5	0
6	7.5	B_2	136	2.04	3.45	29.5	50.0	0
6	25	G_1	103	2.92	9.85	14.2	47.8	0
6	7.5	G_2	140	1.81	4.39	25.5	61.8	0
	65	Total	120					
		Overall	157.6					
Raw peanuts								
6	5	B_1	108	7.52	7.52	171.0	171.0	0
5[b]	5	B_1	68	2.06	2.11	76.3	78.3	1
6	1.5	B_2	146	2.51	2.51	167.6	167.6	0
6	5	G_1	111	7.37	7.37	167.6	167.6	0
5[b]	5	G_1	71	2.04	2.16	72.8	77.1	1
6	1.5	G_2	141	2.43	2.43	173.4	173.4	0
	13	Total[b]	107					
6	10	B_1	101	0.96	2.71	12.6	35.7	0
6	3	B_2	118	0.74	2.24	24.6	74.7	0
6	10	G_1	89	2.38	4.24	35.5	63.3	0
5[b]	10	G_1	69	0.63	1.77	12.1	34.0	1
6	3	G_2	112	0.91	3.41	32.4	121.9	0
5[b]	3	G_2	60	0.34	1.70	22.6	73.2	1
	26	Total[b]	74					
6	25	B_1	87	5.53	5.53	31.8	31.8	0
6	7.5	B_2	120	1.67	2.69	27.8	44.9	0
6	25	G_1	76	6.52	6.54	42.9	43.0	0
6	7.5	G_2	102	1.96	4.38	38.4	85.9	0
	65	Total	96					
		Overall	92.3					

[a] Average natural aflatoxin B_1 contamination levels ("0" columns for all commodities, Table 1) found by collaborators for corn, peanut butter, and raw peanuts were 0.0, 2.2, and 0.21 ng/g, respectively; calculated recovery values do not take into account this source of aflatoxins.
[b] Outlier(s) removed.
[c] Overall average recovery values include outliers.

aqueous phase to the second separatory funnel. The hexane fraction obtained was discarded. The second laboratory that experienced emulsion problems collected the emulsion with the aqueous layer and observed that the emulsion broke during chloroform extraction.

The collaborative data were statistically analyzed to determine or confirm outliers (Dixon test), and compute repeatability (RSD_r) and reproducibility (RSD_R) (8) (Tables 2 and 3). There was good overall agreement between results reported by participating laboratories using both methods for the quantita-

tive step. The reported RSD_r and RSD_R values are comparable but a little higher than those previously obtained for corn (9) and peanut products (7, 10) using the CB and BF (peanut butter only) methods where CV values ranged from 31.8 to 42.7% and from 51.6 to 60.9% for corn and from 16.0 to 68.0% and from 26.0 to 64.0% for peanut butter at spiked concentrations of <13 and 15–30 ng aflatoxin B_1/g, respectively. These studies showed the greatest variation in samples containing <13 ng total aflatoxins/g.

Average percent recovery values for the densitometric and visual method results are presented in Tables 2 and 3, respectively. Overall recovery values observed for densitometry and comparison to standards techniques were 95.3 and 111.0% for corn, 139.0 and 157.6% for peanut butter, and 95.6 and 92.3% for raw peanuts, respectively. Recovery values generally decreased with an increase in aflatoxin contamination levels for peanut butter.

For the current study, reported aflatoxin levels for the nonspiked zero test portions were probably due to the natural variation in aflatoxin contamination in individual nuts (11–14). Although these test portions were ground to pass a U.S. No. 20 sieve for the raw peanuts or prepared as a paste for peanut butter samples, this natural contamination phenomenon probably accounts for most of the variation observed in this set of samples and samples containing low levels of aflatoxins. The blank peanut butter sample seemed to be definitely contaminated and may better explain the high recoveries.

Recommendation

On the basis of the results obtained in this AOAC/IUPAC collaborative study, the Associate Referee recommends that the solvent-efficient TLC method using densitometry for the quantitative step be adopted first action for the determination of aflatoxins B_1, B_2, G_1, and G_2 in corn for levels of 5–50 ng/g, 3–15 ng/g, 10–50 ng/g, and 3–15 ng/g, respectively, by densitometry; for aflatoxins B_1 and B_2 in raw peanuts at 5–25 ng/g and 1.5–7.5 ng/g, respectively, by densitometry; and for aflatoxins B_1 and G_1 in corn at 10–50 ng/g and 50 ng/g, respectively, and aflatoxins B_1, B_2, and G_1 in raw peanuts at 10–25 ng/g, 7.5 ng/g, and 10–25 ng/g, respectively, by visual comparison.

Acknowledgments: Joint AOAC/IUPAC Study

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FOOD CHEMICAL CONTAMINANTS

Determination of Volatile Organic Contaminants in Bulk Oils (Edible, Injectable, and Other Internal Medicinal) by Purge-and-Trap Gas Chromatography/Mass Spectrometry

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Purge-and-trap gas chromatography/mass spectrometry is evaluated for the quantitation of part-per-billion levels of volatile organic contaminants in bulk vegetable oils. Results using 2 purge techniques (direct purging of the heated oil and purging after dispersing the oil on an aluminum oxide powder) and 2 quantitative methods (standard curve and deuterium-labeled internal standard addition) are reported. Twenty volatile compounds and 8 vegetable oils were investigated. Recovery data and estimated detection limits for each compound are reported for each purge technique. Generally acceptable recoveries (70–130% for more than 90% of the analyte spikes) and acceptable detection levels (approximately 4–10 ppb) were obtained for all compounds using either the external standard curve or the deuterium-isotope-labeled internal standard. The use of a dispersant (such as alumina) for sample purging resulted in poor recoveries of the highly volatile contaminants.

Bulk vegetable oils may become contaminated with volatile organic chemicals in various ways. Inadequate cleaning of previous cargos from tanker trucks and ships, permeation of the chemical contaminant through plastic storage or holding containers, and the use of degreasing solvents for cleaning equipment are only a few of the potential sources for such contamination. Health authorities are concerned because many of these potential contaminants are either suspected or proven carcinogens.

Most of these volatile contaminants are highly soluble in the vegetable oils they contaminate. The contaminants that boil at higher temperatures (80°C and above) are difficult to remove efficiently because of their high degree of solubility in oil. In addition to the problem of contaminant solubility in the oil, most of these edible oils contain varying amounts of naturally occurring volatiles that are swept out and trapped during any purge-and-trap procedure. When these naturally occurring volatiles are desorbed into a gas chromatograph with flame ionization detection (FID), a complicated chromatogram with numerous peaks results.

Sensitive and specific analytical techniques are necessary to detect low levels (ppb) of these potential contaminants. Purge and trap with thermal desorption into a gas chromatograph provides adequate concentration and sensitivity. However, in many cases, the flame ionization detector may not be very specific due to interferences from the naturally occurring volatiles in the vegetable oil. Electron capture detection offers greater specificity only for the halogenated contaminants. Gas chromatography/mass spectrometry (GC/MS) coupled with purge-and-trap thermal desorption offers sensitivity and specificity for both halogenated and nonhalogenated contaminants.

Several papers have been reported concerning the determination of volatile organic compounds in foods and food packaging, as well as the evaluation of various purge and trap techniques. McCown and Radenheimer (1) reported on an equilibrium headspace GC method for the determination of volatile residues in vegetable oils and fats. Their procedure involved spiking "prepurified" coconut oil and palm kernel oil with 6 volatile compounds. The "prepurified" coconut and palm kernel oils were prepared (in their laboratory) by heating the oils to 85°C and sweeping with helium for 24 h at 50 mL/min. Quantitation was performed by GC/FID on the equilibrated product headspace. Although their reported detection and recovery levels were very good, prepurifying the oils before spiking resulted in an abnormally clean gas chromatogram (few chromatographic peaks). Many of the naturally occurring volatiles, present in the untreated oils, were swept out in the prepurge purification and did not appear in the chromatogram of the spiked sample (or were present at significantly lower levels in treated oils compared with untreated oils).

Hartman et. al. (2) reported using various GC/MS procedures for the quantitation of purge-and-trap thermal desorption analysis. They concluded that the stable isotope-labeled internal standard method was the most accurate and precise for quantitation.

Overton and Manura (3) qualitatively identified many volatile contaminants and components in various types of liquid products (e.g., water, latex paint, and olive oil) using purge-and-trap thermal desorption GC/MS. No minimum detection levels were indicated, however.

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Kirk and Lehan (4) reported on the determination of volatile organics in dairy products (milk, cheese, and butter) by dynamic headspace/GC. The authors noticed dramatic differences in optimum purge parameters between one type of dairy product and the next. Aerosols formed during the purging process for butter and resulted in carryover contamination from the purging vessel into the trap. As a result of the aerosol problem with butter, their determination was based on a headspace sweep of the heated sample. Milk and cheese also formed aerosols when heated; therefore, these samples were purged at ambient temperature. No minimum detection levels or quantitative results were reported by the authors.

Kirk (5) investigated the flavor qualities of vegetable oils by examining the profiles of volatile organic compounds in the oils at different stages of oxidation. He used small sample sizes heated at 100°C and dynamic headspace GC/MS for the analyses. He reported adequate sensitivity for the range of compounds analyzed but no detection limits. Higher purge temperatures resulted in increased recoveries for all compounds. However, larger sample sizes resulted in an increased carry over from one experiment to the next.

Kester examined the residual solvents and flavoring components in cosmetics (6) and in food packaging materials (7). His qualitative determination indicated the presence of several different types of compounds, including aromatic and aliphatic hydrocarbons and styrene. Although the majority of the compounds detected were naturally occurring components of the oil, a significant number were believed to be the result of packaging adhesives or to be residuals from the package manufacturing process. No quantitative values were reported for any of the compounds detected.

Mosesman et al. (8) evaluated the relative trapping efficiencies of 2 different adsorbent combinations. The authors reported recovery values for 34 different compounds and breakthrough volumes for water and chloromethane by using both the U.S. Environmental Protection Agency (EPA) combination trap (9) and their own experimental trap. They concluded that their experimental trap, containing CARBOPACK B™ and CARBOSIEVE S-III™, was equivalent in recovery efficiency and superior in the amount of retained water to the EPA trap.

Hazard et al. (10) used a steam distillation procedure coupled with purge-and-trap thermal desorption for the determination of hydrocarbons associated with leaking underground storage tanks. They reported a definite correlation between sparging temperature and recovery values for the compounds with higher boiling points. Their highest percent recoveries were obtained using a 12 min sparge at 90°C.

Varner et al. (11) described a method for the determination of benzene in polypropylene food-packaging materials and food-contact paraffin waxes. The polymer was dissolved in hexadecane at 150°C, and the paraffin wax was melted in an 80°C oven. The liquified samples were then purged with helium into a methanol trap. The methanol in the trap was diluted with water, and the heated equilibrated sample headspace was analyzed by GC/FID. Recovery values for benzene in polyethylene were in the 60–70% range, and the limits of detection and quantitation were 8 and 17 ppb, respectively.

Przybylski and Hougen (12) used a chemical reaction trap for the determination of volatile carbonyl compounds in vegetable oils and fried potato chips. Their method involved the use of a nitrogen purge through a tube trap containing hydroxylamine hydrochloride to form the less volatile oxime derivative. Total oximes were then measured at 212 nm and conjugated diene carbonyl oximes at 272 nm.

Keynes (13) reported on the determination of residual hexane in oilseed residues. The hexane was desorbed by heating the sample in a closed vessel at 110°C and measuring the resulting hexane concentration in the equilibrated headspace by capillary or packed-column GC.

Tonogai et al. (14) reported on the determination of several volatile halogenated fumigants in citrus fruits. The fumigants were isolated by distillation with florisil column cleanup and determined by electron capture detection (ECD)/GC.

Entz and Diachenko (15) reported on a procedure for volatile halocarbons in margarines by headspace GC/ECD. They reported levels as high as 1–5 ppm tetrachloroethylene found in margarines obtained from a supermarket located next to a dry cleaning establishment.

Ashley et al. (16) reported on a procedure for determining volatile organic compounds in human blood using purge-and-trap with high resolution GC/MS. They reported part-per-trillion detection levels for 32 volatile organic compounds.

In this study, we determined spike recovery values and detection limits for 20 volatile organic compounds in different types of vegetable oils. Various purging techniques were evaluated, and recovery data and detection limits (based on a minimum signal-to-noise level of 30 to 1) are reported.

Experimental

Apparatus

(**a**) *Purge-and-trap system.*—Tekmar LSC 2000 purge-and-trap instrument with 5 mL needle sparge glassware, stainless steel needle sparge kit, tenax trap, and automatic sample heater. Set the Tekmar purge parameters as follows: sample heater temperature at 90°C with a 5 minute preheat (*Note*: To maintain more uniform heating of the purging vessel, fill the volume between the glass purge tube and the sample heater jacket with aluminum foil); Prepurge time, 0 min; purge time, 11 min; dry purge time, 2 min; desorb preheat, 2 min; desorption temperature, 180°C; desorption time, 4 min; trap bake time, 2 min; and trap bake temperature, 180°C.

(**b**) *Gas chromatograph/mass spectrometer.*—Hewlett-Packard 5890 gas chromatograph with an open-split interface to the Finnigan Model 800 Ion Trap mass selective detector, equipped with an IBM compatible 386 computer. Computer software: Finnigan ION TRAP™ with NIST library for mass spectral data manipulation; QUATTRO PRO™ and PARADOX™ for additional data manipulation and linear-regression analyses of standard curves. GC column: 30 m × 0.25 mm fused-silica open-tubular capillary column bonded with 0.25 μm DB1 liquid phase. GC conditions: helium carrier gas with a linear velocity of 25–30 cm/s; temperature-programmed

run beginning at 30°C for 2 min, then increasing by 12°/min to a maximum of 200°C for 15 min. Set the GC septum purge valve to "off" initially; then set to "on" 30 s into the run. Mass spectrometer conditions: full scan mode with electron impact ionization; electron multiplier voltage, 1800; scan time, 1 s; and filament/multiplier delay set at 100 s.

Reagents

(a) *High purity methanol.*—Burdick and Jackson, distilled in glass, or other equivalent grade.

(b) *Acid alumina.*—Brockman Activity I, 80–200 mesh.

(c) *High purity helium.*—99.5% or better, free from organics.

(d) *Pure oil or prepurified oil.*—For preparation of standard curve. Ideally, this pure oil should be a different lot of the same type of oil as the sample, which has previously been found to be free of the suspected contaminant by the purge-and-trap procedure. If the former oil is unavailable, prepare a prepurified portion of the contaminated oil as follows: Place 75 mL of the oil to be purified into a tapered 100 mL glass centrifuge tube. Place the tube in a uniformly controlled heated water bath maintained at 85–95°C (a hot plate with a magnetic stirrer was found to be adequate). Purge with ca 100 mL/min high-purity helium by using a small glass purge tube with the constricted outlet positioned ca 5–10 mm from the bottom of the tapered end of the centrifuge tube. Purge the oil for at least 10 h, and examine the purified oil using the same purge-and-trap procedure as in the sample determination. The oil is suitable for preparation of the standard curve if the presence of any remaining contaminant is 2% or less of its original amount in the untreated oil. When the oil is suitable for use in preparing the standard curve, sweep the surface of the oil with helium until the oil is cooled to room temperature and stopper it.

(e) *Volatile compound standards.*—All standard compounds (except deuterium-isotope-labeled) were obtained from Supelco, Inc., and were 97% pure or better. All deuterium-labeled standards were obtained from Cambridge Isotope Laboratories and selected from their list of NMR solvents (99% or better in isotopic purity).

Preparation of Standards

(a) *Standard curve stock solution.*—Transfer ca 9 mL of methanol to a 10 mL volumetric flask; stopper and accurately weigh flask. Add separate 50 µL portions of each volatile contaminant standard to the volumetric flask, stoppering the flask, and reweighing it after each standard addition. Dilute to volume with methanol and mix. Calculate the weight of each volatile contaminant standard by difference.

(b) *Diluted standard solution.*—Measure 0, 25, 100, and 150 µL of each standard curve stock solution into separate 10 mL volumetric flasks (or other suitable standard volumes, such that the sample will fall near the middle of the standard curve concentration range). Dilute to volume with methanol and mix.

(c) *Working standard solutions.*—Transfer about 9 mL of pure oil or prepurified oil, reagent (d) above, to each of 4 separate 10 mL volumetric flasks. Add a 50 µL portion of each of the 4 diluted standard solutions to a separate volumetric flask. This procedure results in a series of 0–300 ppb standard solutions for the aromatic organics and 0–600 ppb for the halogenated organics.

(*Note*: Because of the possibility of accidental contamination of samples, prepare all standards and standard dilutions in a different room from that used in the sample handling. Do not use any solvents in the same room as the purge-and-trap apparatus.)

(d) *Deuterium isotope standard solutions.*—Transfer about 9 mL of methanol to a 10 mL volumetric flask; stopper and accurately weigh flask. Add 50 µL of the deuterium isotope standard contaminant to the flask, and stopper and reweigh the flask. Calculate the standard weight by difference.

(e) *Deuterium isotope diluted standard.*—Transfer 50 µL of the stock standard solution to a second 10 mL volumetric flask containing about 9 mL of methanol. Dilute to volume with methanol and mix.

Determination by Direct Purge Standard Curve

Transfer about 9 mL of the sample oil to a 10 mL volumetric flask, add 50 µL of high-purity methanol, dilute flask to volume with sample oil, and mix thoroughly (ca 3 min).

With the 5 mL purging vessel held in a vertical position, weigh 1.0 g of the prepared oil sample into the purging vessel by using a top-loading balance (accuracy, ±0.05 g), without allowing any oil to touch the sides of the tube. Adjust the needle sparge as close to the bottom of the purge tube as possible without restricting the purge gas flow (1–2 mm). As the purging vessel is being attached to the purge instrument, place a small cotton plug around the stainless steel purging needle. This cotton plug should remain in the neck of the purging vessel to help prevent any aerosols formed during the purging process from entering the trap and contaminating the trap and purge lines (Figure 1). Purge and desorb the sample by using the conditions specified in the description of the purge apparatus. Generate selective ion chromatograms of the quantitation ions (Table 1) and determine their area responses. Use these area responses to measure parts per billion of contaminant in the sample directly from the standard curve.

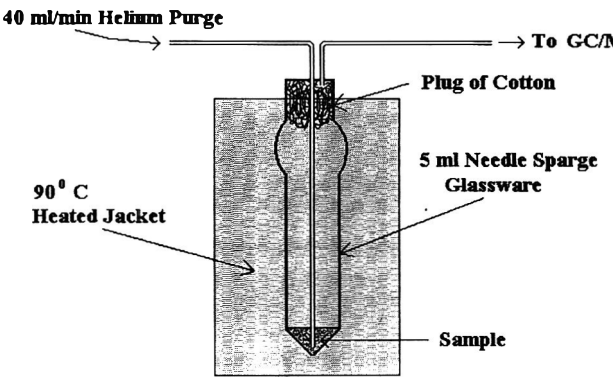

Figure 1. Schematic of the purging vessel arrangement used for the direct purge of the oil sample.

Table 1. Listing of primary and secondary mass spectra quantitation ions, molecular weights, and boiling points

Compound	Primary quant. ion	Secondary quant. ion	MW	bp
Benzene	78	77	78	80
Benzene-d^a	84	83	84	80
Carbon tetrachloride	117	119	152	77
Chlorobenzene	112	77	112	131
Chlorobenzene-d^a	117	82	117	131
Chloroform	83	85	118	62
Chloroform-d^a	84	86	119	62
2-Chlorotoluene	91	126	126	159
Dichlorobenzene	146	111	146	180
Dichlorobenzene-*d*	150	115	150	181
1,2-Dichloroethane	62	98	98	83
1,1-Dichloroethane	63	65	98	57
1,2-Dichloroethene	62	98	96	47
1,2-Dichloropropane	63	112	112	96
1,3-Dichloropropane	76	78	112	121
1,3-Dichloropropene	75	110	110	104
Ethyl benzene	91	106	106	136
Methylene chloride	84	86	84	40
Styrene	104	78	104	146
Tetrahydrofuran	71	72	72	66
Toluene	92	91	92	111
Toluene-d^a	100	99	100	111
1,1,1-Trichloroethane	97	99	132	74
Trichloroethene	95	130	130	87
Vinylidine chloride	96	61	96	32
o-Xylene	106	91	106	144
o-Xylene-d^a	116	101	116	143

[a] Deuterium-isotope-labeled compound.

Purge the previously prepared standards (prepared in pure oil or prepurified oil) in the same manner as the sample. Plot quantitation ion area responses versus concentration for each standard contaminant. A linear-regression analysis of the standard curve data should show a correlation coefficient of 0.98 or better for the best quantitative results.

Calculate the sample concentration from the resulting standard curve. If the sample response does not fall within the standard curve range, adjust the standard concentrations accordingly and repeat the standard curve preparation (alternatively, the sample may be diluted with the purified oil reagent). Linearity has not been established for sample concentrations greater than 600 ppb.

Determination by Direct Purge Deuterium-Isotope-Labeled Internal Standards

Transfer about 9 mL of the oil sample to a 10 mL volumetric flask and add 50 µL of the diluted deuterium-isotope-labeled standard solution. Dilute flask to volume with sample oil and mix thoroughly (ca 30 min). Purge the sample by using the same sample weight and procedure as previously outlined in the determination by "direct purge" standard curve. Generate selective ion chromatograms for the quantitation ions of the sample and the deuterated standard, and determine their area responses. Calculate parts per billion of contaminant in sample by using the following formula:

$$C = \frac{R_{smp}}{R_{std}} P_{std}$$

where C = contaminant in the sample, R_{smp} = area sample response, R_{std} = area deuterated standard, and P_{std} = ppb deuterated standard (within 10 mL volumetric flask).

Determination by Purge from Aluminum Oxide

Add approximately 1 g of aluminum oxide to the 5 mL glass purging vessel, followed by 1.0 g of sample oil. Use care to avoid touching the sides of the vessel with the sample oil. Add an additional 5 g aluminum oxide to the purge vessel above the sample layer. Hold the tube at an angle of about 30° from horizontal on a firm surface. Mix the sample oil and aluminum oxide by using a flat spatula held in the left hand while rolling the tube back and forth between the right hand and the inclined surface. Continue mixing for 1–1.5 min. Attach the purging vessel to the Tekmar instrument with a small cotton plug around the purge needle in the neck of the purge vessel.

Prepare a standard curve (purged from aluminum oxide) in the same manner as the sample above by using the working standard solutions prepared in pure oil reagent.

Results and Discussion

Various trapping adsorbents and adsorbent combinations were evaluated. Tenax GC 60/80, silica gel 15 35/60, activated coconut charcoal, CARBOPACK B, CARBOSIEVE S III, the adsorbent combination trap used in the EPA procedure (9), and the trap used by Mosesman et al. (8) were all examined. No single adsorbent or combination seemed to be superior to the others. Silica gel adsorbed a large amount of water, and the mixed trap used by Mosesman and co-workers could not be consistently reproduced in our laboratory. Tenax GC 60/80 was chosen for the study and gave good consistent recoveries for all compounds evaluated.

Various purging techniques and temperatures were also investigated. Dispersing the oil sample on aluminum oxide (or Celite 545) to increase the surface area gave variable results. The larger particle sizes of the aluminum oxide (60–200 mesh) were much easier to handle and mix with the oil sample than the smaller particle size of Celite 545. Recoveries for the 2 compounds with the lowest boiling points (vinylidine chloride and methylene chloride) were generally very poor and could not be reproduced. Recoveries for the compounds with higher boiling points were generally satisfactory (66–130%). Although the technique may have useful applications, more research is needed to improve reproducibility and the recovery values of the more volatile compounds.

Table 2 summarizes the recoveries obtained after spiking 3 vegetable oils with 20 volatile compounds and purging from the aluminum oxide–oil sample mix.

Table 2. Recovery data obtained from 3 vegetable oils spiked with 20 volatile compounds (1 g sample was mixed with 6 g aluminum oxide prior to purging: quantitation was by standard curve of standard purged from an aluminum oxide mix in the same manner as the oil sample)

Compound	Spike level, ppb	Det. limit, ppb[a]	Rec. from vegetable oil, %			Rec. range, %	Av. rec., %	Av. corr. coef.
			Olive	Peanut	Corn			
Benzene	110	0.4	101	106	81	81–106	96	0.998
Carbon tetrachloride	199	1.8	95	98	80	80–98	91	0.997
Chlorobenzene	138	1.2	93	85	78	78–93	85	0.988
Chloroform	186	0.4	91	101	82	82–101	91	0.990
Chlorotoluene	135	18.6	87	92	79	79–92	86	0.994
1,1-Dichloroethane	156	1.2	97	100	86	86–100	94	0.979
1,2-Dichloroethane	146	0.7	96	104	66	66–104	89	0.996
trans-1,2-Dichloroethene	180	5.6	93	131	70	70–130	98	0.999
1,2-Dichloropropane	145	2.9	94	116	89	89–116	100	0.998
1,3-Dichloropropane	149	1.9	87	115	78	78–115	93	0.998
1,3-Dichloropropene	148	1.3	97	80	79	79–97	85	0.978
Ethyl benzene	110	2.3	100	83	79	83–100	87	0.985
Methylene chloride	166	N/A	0	0	0	N/A	0	N/A
Styrene	114	1.9	81	94	79	81–94	85	0.967
Tetrahydrofuran	111	18.0	75	102	94	75–102	90	0.991
Toluene	110	0.5	124	93	112	93–124	110	0.980
Trichlorothane	185	2.5	98	101	87	87–101	95	0.996
Trichloroethene	185	2.0	83	91	88	83–91	87	0.986
Vinylidine chloride	151	N/A	0	0	0	N/A	0	N/A
o-Xylene	110	2.0	84	92	77	77–92	84	0.994

[a] Detection limit based on a minimum signal-to-noise ratio of 30 to 1.

Directly purging the sample oil from a heated purging vessel was the most reproducible technique evaluated and gave generally good recoveries for all of the spiked contaminants. The 1 g sample size provided adequate sensitivity and tolerable sample matrix interferences.

When no heat was applied during the purging process, the contaminants with higher boiling points showed significantly lower purging efficiencies. Oils purged at too high a temperature resulted in relatively greater interferences from the naturally occurring volatiles in the oils. A compromise temperature of 90°C was found to be satisfactory for adequate recoveries and lower sample matrix interferences.

Figure 2 (A & B) illustrates the purge profiles of 6 compounds, with boiling points ranging from 32 to 159°C, using 2 different purge techniques evaluated in preparing the "prepurified" oil for the standard curve preparation. Figure 2A shows the purge profile resulting from the use of a 125 mL erlenmeyer flask as the purging vessel (without stirring). Figure 2B shows the purge profile when a tapered 100 mL centrifuge tube was used as the purging vessel (also without stirring). Only the compounds with comparatively low boiling points are effectively purged during the first 4 h for either of the 2 purging techniques. The compound with the highest boiling point evaluated in this study (159°C) required almost 9 h to be effectively removed from the oil with the tapered centrifuge tube as purging vessel. This same compound (bp 159°C) was still present in a significant amount (about 8.5% of its original amount) after 31 h using the erlenmeyer flask as purging vessel.

The differences in purge profiles of the different boiling contaminants were essentially nullified by addition of deuterium-isotope-labeled internal standards of the contaminants and by calculation from a standard curve stock solution, purged from the sample oil, as the final diluting solvent.

Figure 3 (A & B) illustrates the powerful selectivity of the mass spectrometer for the determination of volatile contaminants. The total ion chromatogram (Figure 3A) shows numerous extraneous peaks resulting from the sample matrix. The selected ion chromatogram (Figure 3B) for mass-to-charge ratio (m/z) 91 (benzyl or tropylium ion) shows a relatively uncluttered chromatogram (few peaks). Quantitation from the selected ion chromatogram in Figure 3B would be less affected by interferences than the one in Figure 3A.

Table 3 summarizes the recovery results obtained for 20 potential contaminants by direct purge of the oil; calculations were performed with an external standard curve prepared in the "prepurified" sample oil or a related oil product. Eight vegetable oils each were spiked at 100–200 ppb with 20 volatile contaminants. These contaminants had boiling point ranges of 32–159°C. Recoveries ranged from 55 to 162% and were generally satisfactory, with a few exceptions (Table 3). Correlation coefficients of the standard curves ranged from poor to very good (approximately 0.832–1.0), and the majority of the values were 0.99 or better. The majority of the correlation coefficients and

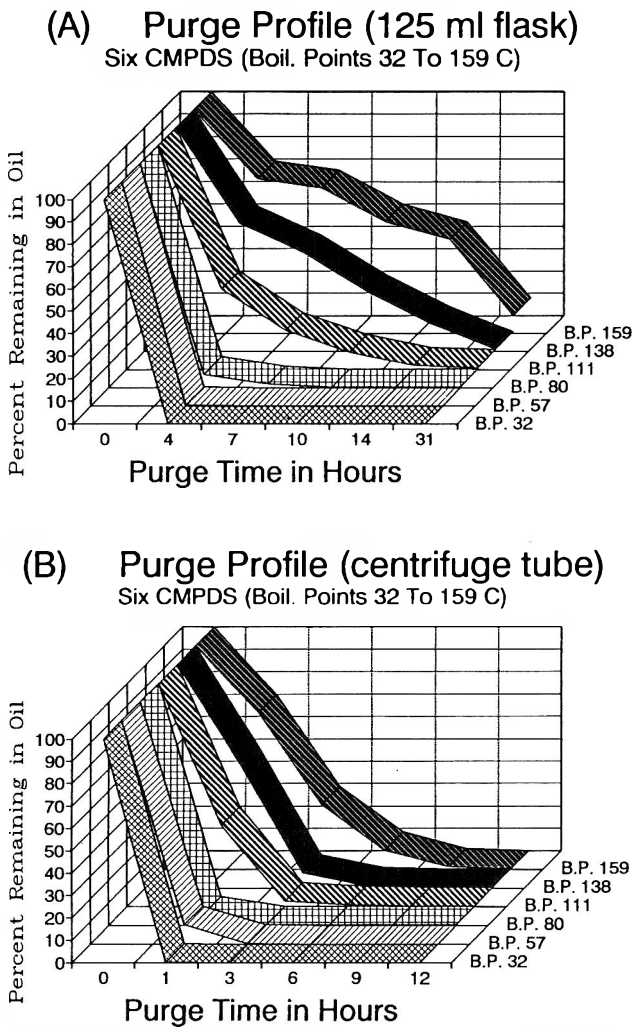

Figure 2. Purge profile of 6 volatile compounds using 2 different purging techniques. (A) Compounds purged with helium from 125 mL erlenmeyer flask at 85°C and no stirring. (B) Compounds purged from a 100 mL tapered centrifuge tube at 85°C with the purge line adjusted to within 1–2 mm of the bottom of the tapered tube and no stirring.

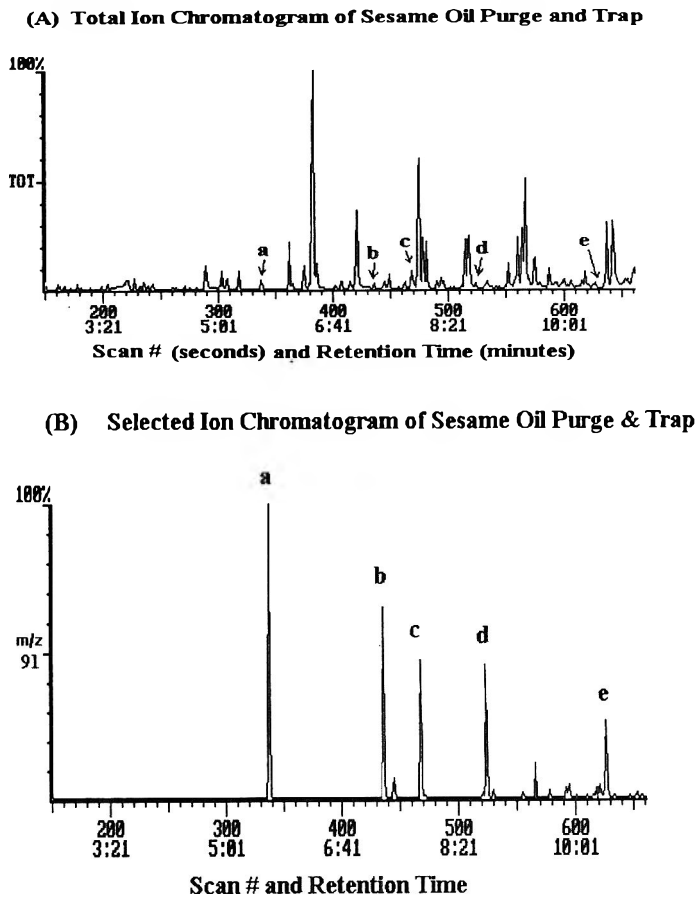

Figure 3. Comparison of the total ion chromatogram (A) to the 91 *m/z* selected ion chromatogram (B) of sesame oil purge and trap. The volatile contaminants in the sample are as follows: a, toluene; b, ethyl benzene; c, xylene; d, chlorotoluene; and e, unknown.

recoveries were considered acceptable for the determination of a broad range of volatile contaminants at part-per-billion levels in vegetable oils.

Table 4 summarizes the recovery data obtained by using deuterium-labeled internal standards. Eight different vegetable oils were spiked with 6 volatile contaminants with boiling points ranging from 62 to 180°C. Recoveries for the 6 compounds varied from 75 to 126% at spiking levels ranging from 55 to 93 ppb. One recovery value for toluene (156%) was discarded because of apparent contamination of the sample (a high toluene background level was found in this sample blank).

One critical part of this purging procedure was the temperature control of the purging vessel during the purging process. A heated jacket was used in this study that required a heating/cooling cycle with each purge-and-trap determination. Reproducing this heated-jacket temperature cycle was considered to be a significant weak point in the overall procedure. Any effect of variation in purge temperature was probably minimized when using the deuterium-labeled internal standard, but temperature variations may have had a significant effect on the external standard curve. A constant-temperature silicone–oil bath would probably have given more reproducible recoveries and higher correlation coefficients. Oil bath heating was not readily feasible during this study.

Calculations of contaminant levels from a standard addition curve were unacceptable because of inconsistency in recoveries and poor correlation coefficients. Again, this inconsistency may have been resulted from inadequate control of the purging vessel temperature in the system used.

Conclusions

Low part-per-billion levels of several potential volatile organic contaminants (with boiling points ≤180°C) can be identified and quantitated in vegetable oils using GC/MS purge-and-trap procedures. Purge efficiencies and limits of detection are dependent on the boiling points of the volatile compounds, design of the purging vessels, and purge temperature. The ad-

Table 3. Recovery data for 20 volatile compounds spiked in 8 vegetable oils using direct purge (quantitation by standard curve of standard purged in a similar manner as sample oil)

Compound	Spike level, ppb	Detect. limit, ppb[a]	Rec. from vegetable oils, %								Rec. range, %	Av. rec., %	Av. corr. coef.
			Olive	Peanut	Corn	Soybean	Saf-flower	Sun-flower	Sesame	Canola			
Benzene	110	1	66	81	83	83	79	79	108	100	66–108	85	0.984
Carbon tetrachloride	199	1	88	90	88	92	84	100	93	122	84–122	95	0.988
Chlorobenzene	138	1.5	115	100	96	94	78	117	107	95	78–117	100	0.995
Chloroform	186	1	105	91	83	93	70	55	115	104	55–115	90	0.967
2-Chorotoluene	135	1.2	120	106	103	87	82	119	100	75	75–120	99	0.994
1,2-Dichloroethane	156	3.3	129	91	93	96	81	58	100	84	58–129	92	0.971
1,1-Dichloroethane	146	3.3	135	91	117	126	83	80	91	94	80–135	102	0.968
1,2-Dichloroethene	180	2.2	112	115	113	100	101	93	103	107	93–115	106	0.979
1,2-Dichloropropane	145	3.1	87	92	91	85	72	75	99	70	70–99	84	0.982
1,3-Dichloropropane	149	2.1	98	102	93	84	72	116	106	86	72–116	95	0.995
1,3-Dichloropropane	148	2.4	100	101	91	83	87	114	120	90	83–120	98	0.989
Ethyl benzene	110	0.7	117	102	104	85	78	126	101	90	78–126	100	0.994
Methylene chloride	166	2.2	110	114	86	106	86	88	79	150	79–150	102	0.99
Styrene	114	2	126	98	93	89	80	101	121	95	80–126	100	0.995
Tetrahydrofuran	111	7.6	130	116	92	67	87	78	103	98	67–130	96	0.985
Toluene	110	0.4	84	96	92	89	81	127	111	92	81–127	97	0.995
1,1,1-Trichloroethane	185	0.8	122	88	93	92	79	76	106	125	76–125	98	0.987
Trichloroethene	185	3	84	89	94	94	79	95	58	96	58–96	86	0.996
Vinylidine chloride	151	1.8	93	92	86	96	72	76	64	162	64–162	93	
o-Xylene	110	2.9	127	111	108	84	80	137	74	75	74–137	100	
Min. rec., %			66	81	83	67	70	55	58	70			
Max. rec., %			135	116	117	126	101	137	121	162			
Av. rec., %			107	98	95	91	81	96	98	101			
Av. rec. RSD, %			18	10	10	13	9	25	18	23			
Av. min. det. level, ppb			4	6	4	6	5	8	9	7			
Av. corr. coef.			0.994	0.991	0.995	0.993	0.995	0.965	0.994	0.971			

[a] Detection limit based on a minimum signal-to-noise ratio of 30 to 1.

Table 4. Recovery data for 6 compounds in 8 vegetable oils using deuterium-labeled internal standard addition

Vegetable oil	Volatile compound					
	Chloroform	Benzene	Toluene	Chlorobenzene	Xylene	Dichlorobenzene
Olive	75	98	90	103	101	126
Peanut	86	90	98	93	112	115
Corn	95	89	99	89	103	83
Soybean	87	99	103	90	104	99
Safflower	87	105	85	89	108	107
Sunflower	91	92	105	88	84	106
Sesame	90	95	156[a]	105	91	96
Canola	91	101	89	90	99	113
Spike level, ppb	93	55	55	69	55	82
Av. rec., %	88	96	96	93	100	106
Min. rec., %	75	89	85	88	84	83
Max. rec., %	95	105	104	105	112	126
Rec. RSD, %	6.7	5.8[a]	7.8	7.2	9.0	12.4

[a] Value not included in average recovery, %RSD, or min-max values (apparent contamination of the sample).

dition of a plug of cotton in the neck of the purging vessel reduces the amount of aerosol carryover of the purged oil into the trap. Twenty or more determinations were made in this study without significant increases in system background when using the cotton plug.

The use of a solid dispersant for the oil sample (aluminum oxide) was unsatisfactory because of very poor recoveries of the highly volatile contaminants. Moreover, the purge efficiencies of the contaminants with higher boiling points did not appear to be significantly improved by using a dispersant instead of direct purging.

Mass spectral confirmation of analyte identity can be obtained with the reported method. Full-scan mass spectra were obtained for all compounds studied at each spiking level.

Recovery values were satisfactory (more than 90% of the analyte spikes were within 70–130%) with quantitation by either the external standard curve or by the deuterium-labeled internal standard at spiking levels of 55–200 ppb. Better recoveries with less variability were obtained with the deuterium-labeled internal standards. If appropriate standards are available, this appears to be the quantitative method of choice.

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FOOD CHEMICAL CONTAMINANTS

Enzyme-Linked Immunosorbent Assay of Total Aflatoxins B_1, B_2, and G_1 in Corn: Follow-up Collaborative Study

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A direct competitive enzyme-linked immunosorbent assay screening method for aflatoxins at 20 ng/g in corn was studied by 15 collaborating laboratories. Test samples of corn were extracted by blending with methanol–water (8 + 2). The extracts were filtered and the filtrates were diluted with buffer to a final methanol concentration of <30%. Each diluted filtrate was applied to a test device containing a filter with immobilized polyclonal antibodies specific to aflatoxins B_1, B_2, and G_1. Aflatoxin B_1-peroxidase conjugate was added, the test device was washed with water, and a mixture of hydrogen peroxide and tetramethylbenzidine was added. A test sample was judged to contain ≥20 ng aflatoxins/g when, after exactly 1 min, no color was observed on the filter; if a blue or gray color developed, the test sample was judged to contain <20 ng aflatoxins/g. All laboratories correctly identified naturally contaminated corn test samples. Only one false positive was found for controls containing no aflatoxins. The correct responses for positive test samples spiked at levels of 10, 20, and 30 ng aflatoxins/g (the ratio of B_1:B_2:G_1 was 15:1:3) were 67, 97, and 100%, respectively. This method was adopted first action by AOAC INTERNATIONAL as a change in method for 990.34 for screening for aflatoxins B_1, B_2, and G_1 in corn at total aflatoxin concentrations of ≥20 ng/g.

Submitted for publication November 11, 1992.

The recommendation was approved by the Committee on Natural Toxins, and was adopted by the Official Board of the Association. See "Changes in Official Methods of Analysis" (1994) *J. AOAC Int.* **77**, Jan/Feb issue, and "Official Methods Board Actions" (1993) *The Referee*, **17**, July issue.

This study was carried out under the joint sponsorship of AOAC INTERNATIONAL and the Commission on Food Chemistry of the International Union of Pure and Applied Chemistry (IUPAC).

Simple, specific, sensitive enzyme-linked immunosorbent assays (ELISA) have been used to supplement or replace the traditional thin-layer chromatographic screening methods for aflatoxins in foods and agricultural commodities. The typical direct competitive ELISA format for aflatoxins contains 3 specific reagents: *(1)* the mono- or polyclonal antibodies that recognize the aflatoxins and bind with them; *(2)* an aflatoxin-enzyme conjugate (usually aflatoxin-horseradish peroxidase); and *(3)* an enzyme substrate, such as hydrogen peroxide and tetramethylbenzidine. Binding of the aflatoxin-enzyme conjugate by immobilized antibodies is inhibited by the presence of free toxin in the test sample. The bound enzyme catalyzes oxidation of the substrate to form a colored complex. Development of color indicates that the test sample contains no aflatoxin or an aflatoxin concentration below the level of interest. A screening method, which permits the analysis of a large number of test samples at the designated level of interest, is used to eliminate (screen) negative test samples (1).

Several ELISA methods for aflatoxins have been adopted first action by AOAC (2, 3). One method, **990.34**, was approved for screening cottonseed and peanut butter for aflatoxins at ≥20 ng/g and corn and raw peanuts for aflatoxins at ≥30 ng/g after collaborative study (4). Recently, this method was modified because the dimensions of the test device were reduced. Subsequently the volumes of the test extract and reagents were also reduced. The purpose of this collaborative study was to assess the capacity of the modified method to correctly classify corn test samples as either positive or negative, at a predetermined level (20 ng/g) of aflatoxins. The other commodities (cottonseed, peanut butter, and raw peanuts) will be evaluated in an in-house study by the manufacturer of the test device. The protocol for this collaborative study was approved by AOAC. The results of the study are reported here.

Collaborative Study

Portions of 55 kg ground corn were analyzed according to **968.22** and found to contain <1 ng aflatoxins/g. Test samples were spiked in duplicate with total aflatoxins B_1, B_2, and G_1 at 30, 20, and 10 ng/g. The ratio of B_1:B_2:G_1 was 15:1:3. The ar-

tificially contaminated (spiked) test samples, controls (<1 ng/g), naturally contaminated corn (23 ng/g) test sample (analyzed according to **968.22**), 2 positive practice test samples (30 ng/g), and 1 negative practice test sample (<1 ng/g) were sent to 16 collaborating laboratories in the United States and France. Reference standards, test kits, and method directions were also provided.

Statistical Parameters

Confidence intervals of 95% were obtained by using the binomial distribution test and by treating each duplicate result from 15 laboratories as a single determination. The conventional within-laboratory and among-laboratories parameters are not applicable to either/or, qualitative, or spot tests.

993.16 Total Aflatoxins (B_1, B_2, and G_1) in Corn—Enzyme-Linked Immunosorbent Assay Method (Afla-20 Cup Test)

AOAC-IUPAC Method

First Action 1993

(Applicable to detection of ≥20 ng total aflatoxins/g in corn.)

Method Performance:

The 95% confidence interval for correct identification of corn test samples positive for aflatoxin contamination at 20 ng/g level in the collaborative study was 83–100%.

See Table **993.16** for method performance data.

(*Caution*: Aflatoxins are carcinogenic in some animals. *See Appendix: Laboratory Safety*, section on carcinogens and solvents, for safe handling.)

A. Principle

See **990.34A**.

B. Antibody Specificity

Cross-reactivities of polyclonal antibody used in the collaborative study of this method were 100, 70, 75, and <10% for aflatoxins B_1, B_2, G_1, and G_2, respectively.

C. Sensitivity of ELISA Reagent

See **990.34D**.

D. Reagents

(**a**) *Antibody-coated solid support.*—Aflatoxin antibody-coated porous polyethylene membrane, with 0.71 cm diam. opening, over layer of adsorbent material; support device consisting of high-density polyethylene housing (3.4 cm diam. × 2.65 cm ht) with centered cone-shaped ring insert (3.09 cm diam. × 0.9 cm ht) (Immunodot Screen Cup, International Diagnostic Systems Corp. (IDS), PO Box 799, St. Joseph, MI 49085).

See **990.34*E*(b)-(h)**.

E. Apparatus

See **990.34*F***.

F. General Instructions

See **990.34*G***.

G. Extraction of Test Sample

See **990.34*H***.

H. Preparation of Aflatoxin B_1 Standard Solutions

(**a**) *Stock solution.*—Dissolve 25 µg dry film aflatoxin B_1 in 2.5 mL methanol (10 ng/µL). Store at <–20°. In absence of acid or base, solution is stable for ca 6 months in clean container.

(**b**) *Working standard.*—Dispense 250 µL stock solution into clean vial. Add 2250 µL methanol and mix. May be stored 1 month at ca 5° (1 ng/µL).

(**c**) *Buffer solution of standard.*—Prepare <2 h before use. Dispense 5 µL working standard, (**b**), into test tube. Add 300 µL methanol and 700 µL buffer, *E*(**d**), and agitate vigorously. Proceed as for diluted test extract, **990.34*J*(a)***(4)***.

I. Enzyme Immunoassay

[*Note*: Allow 1 h for reagents to reach room temperature (20–23°).]

Prepare fresh substrate in small tube by mixing 500 µL (10 drops) substrate solution A, **990.34*E*(e)**, with 500 µL (10 drops) substrate solution B, **990.34*E*(f)**, for each reaction site used.

Add 100 µL test extract, **990.34*H*(a)**, to 200 µL buffer (total volume, 300 µL) and thoroughly mix. Apply 100 µL diluted test extract to center of membrane. **993.16*D*(a)**. Using timer, wait 1 min.

Apply 100 µL (2 drops) enzyme solution, **990.34*E*(b)**, to center of membrane. Using timer, wait 1 min. Wash 1× with 1500 µL (30 drops) wash solution, **990.34*E*(c)**, added dropwise. If >1 reaction site is used, wash successively with 10 drops per site, 3×.

Add entire substrate solution, 1000 µL (20 drops), from each tube to each reaction site. Using timer, wait 1 min from addition of substrate mixture to reaction site and immediately observe site for blue color development (negative) or no color development (positive).

J. Interpretation of Results

See **990.34*K***, except *(1)* method now detects aflatoxins in corn at ≥20 ng/g and *(2)* add that positive test samples should be confirmed by quantitative method, such as **991.31**.

Ref.: JAOAC **74**, 81–88 (1991)

Results and Discussion

Of the 16 potential collaborating laboratories, 15 completed the study and submitted data. One laboratory did not perform the analyses because the package of samples had been stored at room temperature for more than a month. The results are presented in Table 1. No outlier test was applied; however, it was apparent that the data were internally consistent. The replicates were treated as independent measurements. In computing the confidence limits, even though 15 laboratories provided

Table 993.16 Method Performance for Immunosorbent Assay Method for Aflatoxins B_1, B_2, and G_1 in Corn

Total aflatoxins, ng/g	Positive assays at each level	% Positive	Lower bound[a]	Upper bound[a]	Sensitivity rate, %	Specificity, rate, %
0	1	3.3	0.1	17.2	–	96.7
10	20	66.7	47.2	82.7	66.7	–
20	29	96.7	82.8	99.9	96.7	–
30	30	100.0	90.5	100.0	100.0	–
NC[b]	30	100.0	90.5	100.0	100.0	–

[a] Lower and upper bound of % positive assays.
[b] NC = naturally contaminated at 23 ng/g.

2 estimates each, the results were treated as if they came from 30 laboratories, each providing a single estimate. The 95% confidence interval was obtained by using the binomial distribution. For this study, the 95% confidence interval of the % positive rate for detecting aflatoxin contamination at 20 ng/g was 82.8–99.9%. Table 1 shows the confidence intervals for all levels tested. All laboratories correctly identified naturally contaminated corn test samples. Only 1 false positive was found for controls containing no aflatoxins. The % correct responses for positive test samples spiked at levels of 10, 20, and 30 ng aflatoxins/g were 66.7, 96.7, and 100%, respectively.

Figure 1 shows the operating characteristic (OC) curve of results of this collaborative study. The OC curve is a plot of the true positive rate (positive at target level) or percentage positive as a function of concentration. The false positive rate (positive at levels below the target level) is also a function of concentration. The performance of this study is characterized by a high true positive rate (<95%) at the target level (20 ng/g).

Collaborators' Comments

Comments from the collaborators indicated that the test was a quick and simple way to test for aflatoxins in corn at ≥20 ng/g.

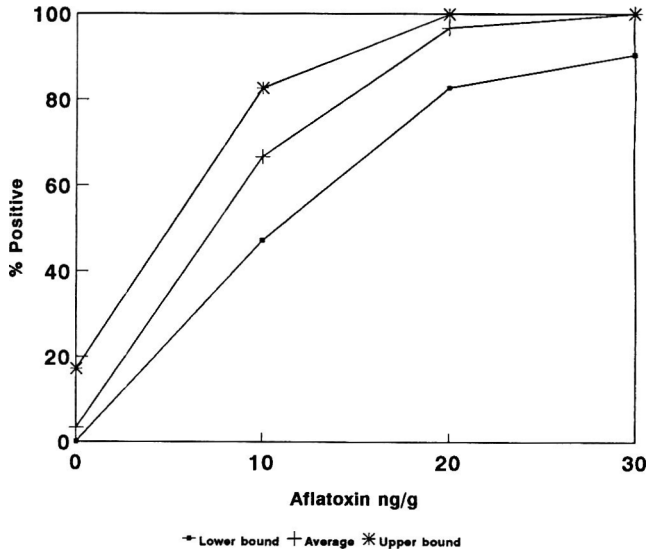

Figure 1. Operating characteristic curve of ELISA method for aflatoxins in corn.

Recommendation

On the basis of the results obtained in the AOAC/IUPAC collaborative study reported here, the Associate Referee recommends that the ELISA method be adopted first action as a change in AOAC method **990.34** for screening for aflatoxins B_1, B_2, and G_1 at ≥20 ng/g in corn. Positive test samples should be reanalyzed by an official, quantitative method, such as AOAC method **991.31**.

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Table 1. Collaborative results for ELISA method for screening for aflatoxins B_1, B_2, and G_1 in corn[a]

Total aflatoxins, ng/g	Laboratory															Positive assays at each level	% Pos.	Lower bound	Upper bound
	1	3	4	5	6	7	8	9	10	11	12	13	14	15	16				
0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0				
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	3.3	0.1	17.2
10	1	1	1	1	1	1	1	1	0	1	0	0	0	1	1				
10	1	1	1	1	1	0	1	1	0	0	1	0	1	0	0	20	66.7	47.2	82.7
20	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1				
20	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	29	96.7	82.8	99.9
30	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1				
30	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	30	0.0	90.5	100.0
NC	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1				
NC	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	30	0.0	90.5	100.0
Sum	8	8	8	8	8	7	8	8	7	7	7	5	7	7	7				

[a] 0 = negative; 1 = positive; NC = naturally contaminated at 23 ng/g.

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FOOD CHEMICAL CONTAMINANTS

Predicting the Distribution of Aflatoxin Test Results from Farmers' Stock Peanuts

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Suitability of the negative binomial function for use in estimating the distribution of sample aflatoxin test results associated with testing farmers' stock peanuts for aflatoxin was studied. A 900 kg portion of peanut pods was removed from each of 40 contaminated farmers' stock lots. The lots averaged about 4100 kg. Each 900 kg portion was divided into fifty 2.26 kg samples, fifty 4.21 kg samples, and fifty 6.91 kg samples. The aflatoxin in each sample was quantified by liquid chromatography. An observed distribution of sample aflatoxin test results consisted of 50 aflatoxin test results for each lot and each sample size. The mean aflatoxin concentration, m; the variance, s_x^2 among the 50 sample aflatoxin test results; and the shape parameter, k, for the negative binomial function were determined for each of the 120 observed distributions (40 lots times 3 sample sizes). Regression analysis indicated the functional relationship between k and m to be $k = 0.000006425m^{0.8047}$. The 120 observed distributions of sample aflatoxin test results were compared to the negative binomial function by using the Kolmogorov–Smirnov (KS) test. The null hypothesis that the true unknown distribution function was negative binomial was not rejected at the 5% significance level for 114 of the 120 distributions. The negative binomial function failed the KS test at a sample concentration of 0 ng/g in all 6 of the distributions where the negative binomial function was rejected. The negative binomial function always predicted a smaller percentage of samples testing 0 ng/g than was actually observed. However, the negative binomial function did fit the observed distribution for sample test results at a concentration greater than 0 in 4 of the 6 cases. As a result, the negative binomial function provides an accurate estimate of the acceptance probabilities associated with accepting contaminated lots of farmers' stock peanuts for various sample sizes and various sample acceptance levels greater than 0 ng/g.

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The peanut industry is interested in developing an aflatoxin testing program that would classify farmers' stock peanut lots into different categories according to the lot's aflatoxin concentration. Previous studies demonstrated the feasibility of replacing the present visual *Aspergillus flavus* method for inspecting farmers stock peanut lots with a chemical testing program at the buying point (1). Companion studies also were developed to determine the variability associated with testing farmers' stock peanut lots for aflatoxin when using 2.3, 4.2, and 6.9 kg sample sizes (2). In addition to sample variabilities, the variability associated with sample preparation and analytical steps of the aflatoxin testing procedure were also determined.

To develop an effective aflatoxin testing program, an evaluation method based on statistical principles needs to be developed. A statistically based evaluation method would provide the peanut industry with the tools to evaluate proposed aflatoxin testing plans and determine if the plans meet the criteria specified by the peanut industry. An evaluation method should be able to predict for any aflatoxin testing plan the following

characteristics: (*1*) the probability that a lot with a given aflatoxin concentration will either be accepted or rejected, (*2*) the percentage of lots misclassified (either false positives or false negatives), (*3*) the percentage of lots removed from the marketing chain, (*4*) either the amount of aflatoxin removed from or the amount of aflatoxin remaining in the marketing chain, and (*5*) the cost of the testing program.

The basic component of an evaluation method is the probability distribution function that can predict accurately the distribution of replicate sample aflatoxin test results taken from a contaminated lot of farmers' stock peanuts. The probability distribution function should be able to predict the distribution of sample aflatoxin test results for any sample size, any sample preparation technique, any subsample size, and any analytical method. An evaluation method was developed and used by the peanut industry to design aflatoxin-testing plans for shelled peanuts (3–6). The evaluation method for shelled peanuts was based on the negative binomial probability distribution function, which was shown to simulate accurately the distribution of observed sample aflatoxin test results taken from a contaminated lot (4).

Because the negative binomial function adequately fitted the observed distribution of sample aflatoxin test results for shelled peanuts, it was decided to compare the same function with the observed distribution of sample aflatoxin test results for farmers' stock peanuts. However, new distribution parameters that reflect the testing of farmers' stock peanuts for aflatoxin need to be developed. The objectives of this study were to (*1*) determine the parameters of the negative binomial function from observed distributions of sample aflatoxin test results, (*2*) compare the negative binomial function with the observed distribution of sample aflatoxin test results and determine goodness of fit, and (*3*) demonstrate how the evaluation method can be used to predict the operating characteristic curve for a given aflatoxin-testing plan used to classify a farmer's peanut lot.

METHOD

Theoretical Distribution

Whitaker and Wiser (3), in previous studies on shelled peanuts, described reasons for choosing the negative binomial function to simulate the distribution of sample test results. The negative binomial function is

$$F(X) = \left(\frac{\Gamma[X+K]}{[X!\Gamma(K)]}\right) p^K q^X \qquad (1)$$

for $X = 0, 1, 2, \ldots$, where X is the quantity of aflatoxin on a single peanut pod, $p = [K/(K+M)]$, $q = [M/(M+K)]$, M is the average quantity of aflatoxin in the total population (lot), K is a shape parameter, and Γ is the gamma function. When each peanut pod in the lot is assumed to weigh the same, X denotes aflatoxin concentration (ng/g) in each pod, and M denotes the average concentration of aflatoxin in the lot.

If the random variable X has a negative binomial distribution with parameters M and K, then the distribution of the sum of N independent observations is negative binomial with mean NM and shape parameter NK (4). The ΣX_i is equivalent to $N\bar{X}$, where $\bar{X}$ is the concentration of aflatoxin in the sample and N is the number of pods in the sample. Therefore, the cumulative distribution of the sum of N observations is

$$C(N\bar{X}) = \sum_{r=0}^{N\bar{X}} \left(\frac{\Gamma\{r+NK\}}{\{r!\Gamma(NK)\}}\right) p^{NK} q^r \qquad (2)$$

where r is a summation index for the variable for $N\bar{X}$. The cumulative distribution of the aflatoxin concentrations in the samples $C(\bar{X})$ can be determined by a scale transformation from equation 2. For given M and K values, equation 1 describes the distribution of pods according to their aflatoxin concentration within a lot and equation 2 describes the distribution of replicate sample aflatoxin test results taken from a lot.

Observed Distribution

Forty farmers' stock lots (averaging about 4100 kg or 9000 lbs each) of runner peanuts suspected of containing aflatoxin were identified by the Federal State Inspection Service by visual inspection during the 1990 crop season. It was assumed that these 40 lots were typical of contaminated runner peanut lots produced in the United States. A 900 kg (1982 lb) portion was removed from each of the 40 lots by using a divider as the peanuts were being unloaded. By using another specially constructed divider, each 900 kg portion was divided into 64 samples of 2.27 kg (5 lb), 64 samples of 4.54 kg (10 lb), and 64 samples of 6.81 kg (15 lb). Approximately 50 samples of each size for each lot, or a total of about 6000 samples, were tested for aflatoxin. Some of the remaining 14 samples per sample size per lot were used in other studies. From the excess samples, a 4.54 kg sample from each lot was used to estimate the number of pods per kilogram.

Each sample contained all components of a farmer's stock lot, foreign material, loose shelled kernels, and pods. The weight of each component of the sample (foreign material, loose shelled kernels, hulls, and shelled kernels) was recorded. Sample weights in this study excluded foreign material and reflect loose shelled kernels and pods only. For each sample, the foreign material was removed and the pods were shelled. All kernels (loose shelled kernels and shelled kernels were combined) were comminuted in a Stephan vertical cutter mixer (VCM) for 7 min. A 100 g subsample, regardless of sample size, was removed from the comminuted sample. Aflatoxin was extracted from the 100 g subsample with acetonitrile–water (90 + 10, v/v) in a 3/1 solvent volume/peanut weight ratio. The extract was purified through a Mycosep 224 column (Romer Labs, Washington, MO), and aflatoxins were quantified by reversed-phase liquid chromatography (LC) procedures described by Wilson and Romer (7) and Hagler and Whitaker (8). Recoveries with the Mycosep column were greater than 98% when using concentrations up to 200 ng/g. Recoveries were considered to be more of a concern at low concentrations than

Table 1. Average concentration, *m*, among 50 sample aflatoxin test results; variance, $s^2_{\bar{x}}$, among 50 sample aflatoxin test results; average sample size, *n*, in number of pods; and shape parameter, *k*, of the negative binomial function, for each farmers' stock lot; sample sizes averaged 2.26 kg

Lot	Av. aflatoxin, *m*, ng/g	Variance, $s^2_{\bar{x}}$	Number of pods, *n*	Shape parameter, *k*[a]
27	33.8	11010.3	1829.7	0.000057
39	79.0	13362.9	2006.0	0.000233
32	83.2	9664.2	2623.1	0.000273
28	106.3	35079.7	1678.7	0.000192
18	106.5	28843.9	2011.8	0.000195
6	129.2	42894.7	1903.6	0.000204
19	173.9	50399.9	1785.8	0.000336
30	186.4	35669.0	1747.5	0.000558
36	215.2	33892.2	1942.0	0.000704
29	262.0	81828.3	1724.9	0.000486
35	275.8	26757.8	2161.1	0.001315
20	352.3	48676.1	2757.8	0.000924
24	358.2	109329.6	1855.2	0.000632
34	431.6	117180.0	1863.6	0.000853
17	514.7	165450.8	2107.8	0.000760
1	547.3	82529.3	1836.4	0.001976
33	567.3	76884.5	2453.8	0.001706
38	599.3	176686.6	2186.5	0.000930
40	691.8	150767.7	2008.7	0.001580
13	843.7	158133.7	2123.7	0.002120
21	906.5	206285.1	1947.4	0.002045
26	945.7	172424.4	2286.2	0.002269
12	955.2	219437.4	1826.0	0.002277
2	1031.8	163073.8	1790.5	0.003646
22	1072.6	436925.9	1575.8	0.001671
9	1092.4	201885.7	2150.9	0.002748
37	1114.8	307662.7	2381.3	0.001696
8	1126.9	243447.9	1900.6	0.002745
23	1181.7	470343.7	2059.4	0.001441
16	1240.0	304858.6	1767.1	0.002854
31	1753.4	340575.7	2411.1	0.003744
14	1768.3	381287.1	1826.7	0.004489
5	2025.7	546171.8	1683.6	0.004462
3	2548.2	974489.0	2136.9	0.003118
25	2697.9	995046.4	1891.0	0.003868
15	3034.5	763110.3	1981.1	0.006091
11	4656.3	992809.7	2008.8	0.010871
4	7079.5	2100344.6	2017.4	0.011828
7	8124.8	6677496.9	1898.1	0.005208
10	24876.7	7833387.5	1820.3	0.043402

[a] $k = (m \times m) / [(n \times s^2_{\bar{x}}) - m]$.

Table 2. Average concentration, *m*, among 50 sample aflatoxin test results; variance, $s^2_{\bar{x}}$, among 50 sample aflatoxin test results; average sample size, *n*, in number of pods; and shape parameter, *k*, of the negative binomial function, for each farmers' stock lot; sample sizes averaged 4.21 kg

Lot	Av. aflatoxin, *m*, ng/g	Variance, $s^2_{\bar{x}}$	Number of pods, *n*	Shape parameter, *k*[a]
27	31.6	4577.7	3325.7	0.000066
28	46.1	6584.9	3334.5	0.000097
32	50.2	2483.7	4989.0	0.000203
18	64.1	5046.2	4080.3	0.000196
39	100.5	14572.2	3383.4	0.000205
6	124.5	15271.0	3396.2	0.000299
30	158.2	21631.8	3120.5	0.000371
36	225.9	24424.2	3417.8	0.000611
29	236.3	37630.3	3382.6	0.000439
19	250.5	11204.1	3579.0	0.001565
20	253.7	17685.1	4783.0	0.000761
24	295.1	30767.8	3477.0	0.000814
35	318.6	28142.0	3775.6	0.000956
34	432.7	82654.3	3243.6	0.000698
17	502.5	59785.6	3772.8	0.001210
33	582.8	56044.3	4281.0	0.001416
38	594.1	53199.0	3870.4	0.001714
1	651.3	84888.0	3668.1	0.001362
40	715.7	114915.2	3496.3	0.001275
9	893.7	53474.6	4278.9	0.003490
21	961.8	158787.5	3355.0	0.001736
26	982.5	149787.2	3974.4	0.001622
22	985.6	397823.2	3127.7	0.000781
37	1039.1	153766.6	4093.4	0.001715
12	1056.0	197515.3	2979.2	0.001895
13	1067.7	121884.7	4330.8	0.002160
16	1178.8	199861.9	3624.6	0.001918
2	1204.7	139221.1	3383.3	0.003081
23	1271.2	268238.6	3875.1	0.001555
8	1325.6	352967.6	3866.7	0.001288
31	1769.3	319269.8	4602.6	0.002130
14	1841.8	297520.5	3406.3	0.003347
5	2195.1	493512.7	3340.5	0.002923
25	2469.1	382921.3	3524.4	0.004517
15	3018.5	353869.5	3763.3	0.006842
3	3093.3	725203.7	4148.2	0.003181
11	4039.7	1281121.3	4062.2	0.003136
4	7110.7	2345137.9	3937.2	0.005476
7	7683.1	8918263.5	3665.2	0.001806
10	29053.8	7670896.2	3245.7	0.033904

[a] $k = (m \times m) / [(n \times s^2_{\bar{x}}) - m]$.

at high concentrations because of the mechanisms by which the Mycosep column removes interfering compounds. Aflatoxin concentrations are reported as nanograms per gram of total aflatoxin or the sum of aflatoxins B_1, B_2, G_1, and G_2.

Aflatoxin test results were considered to be estimates of sample concentrations $\bar{X}$ and are denoted by $\bar{x}$. Estimates of *M*, *K*, and *N*, based on experimental values, are denoted by *m*, *k*, and *n*, respectively. For each lot and each sample size, an observed distribution consisted of 50 sample aflatoxin test results, $\bar{x}$ and *m* is the average of the 50 $\bar{x}$ values. As a result, there are 120 observed distributions (40 lots times 3 sample sizes per lot).

Table 3. Average concentration, *m*, among 50 sample aflatoxin test results; variance, $s_{\bar{x}}^2$, among 50 aflatoxin test results; average sample size, *n*, in number of pods; and shape parameter, *k*, of the negative binomial function, for each farmers' stock lot; sample sizes averaged 6.91 kg

Lot	Av. aflatoxin, *m*, ng/g	Variance, $s_{\bar{x}}^2$	Number of pods, *n*	Shape parameter, *k*[a]
27	23.7	1274.5	5652.3	0.000078
28	44.4	2663.1	5358.1	0.000138
32	67.6	2950.2	8176.2	0.000190
18	84.6	7143.6	6540.3	0.000153
39	87.4	8371.8	5768.6	0.000158
6	145.4	24613.0	5592.0	0.000154
19	160.3	16404.5	5683.1	0.000276
30	187.7	23877.9	5257.6	0.000281
29	208.8	54552.8	5416.6	0.000148
36	250.8	18076.6	5793.8	0.000601
20	308.8	17758.8	7538.6	0.000712
24	316.4	18310.3	5632.2	0.000971
35	323.3	34583.7	6351.0	0.000476
34	417.5	50775.5	5534.0	0.000620
17	511.3	40564.8	6334.2	0.001017
33	574.0	66820.1	7170.8	0.000688
1	633.9	35166.9	6073.9	0.001881
38	647.4	66698.6	6475.8	0.000970
40	734.2	56456.2	6024.9	0.001585
9	813.1	39384.2	7057.2	0.002379
13	925.7	56284.9	6693.7	0.002275
26	934.9	77549.6	6727.4	0.001675
22	936.6	271156.6	5011.1	0.000646
21	994.4	88555.0	5655.9	0.001974
12	1068.8	167471.9	5107.7	0.001335
23	1143.3	104100.1	6329.2	0.001984
37	1192.4	291768.9	6861.2	0.000710
16	1309.0	133951.1	5631.4	0.002272
2	1345.5	80460.9	5334.7	0.004218
8	1475.2	100466.6	6261.6	0.003460
31	1590.7	132218.6	7587.6	0.002522
5	1733.4	110498.2	5406.3	0.005030
14	1792.6	265032.2	5631.8	0.002153
3	2606.6	268122.9	6664.3	0.003802
25	3180.1	587598.1	5815.3	0.002960
15	3330.0	457256.1	6154.5	0.003941
11	4353.7	489685.0	6557.4	0.005903
4	6972.2	1962790.5	5940.0	0.004169
7	8215.1	13163362.2	5832.6	0.000879
10	20453.2	9102228.8	5282.4	0.008701

[a] $k = (m \times m) / [(n \times s_{\bar{x}}^2) - m]$.

Parameter Estimation

The function in equation 1 has been described in detail by Anscombe (9) and Whitaker et al. (3, 4). Anscombe (9) discussed 5 methods to estimate the parameters M and K in equation 1. The procedure, called the "method of moments," was used in this study.

The first moment of equation 1 is

$$\mu_1 = \frac{Kq}{p} = M \tag{3}$$

and the second moment about the mean is

$$\mu_2 = \frac{Kq}{p^2} = M + \frac{M^2}{K} = \sigma^2 \tag{4}$$

where σ^2 is the variance among pod aflatoxin concentrations in the lot. Equation 4 shows that $\sigma^2 \geq M$ for the negative binomial probability distribution. As K approaches infinity, $\sigma^2 = M$, which is characteristic of the Poisson distribution.

The parameter M is estimated by m or the average of the 50 sample aflatoxin test results in each observed distribution. The variance among the pod aflatoxin concentrations, σ^2, is equal to the variance among the sample concentrations, $\sigma_{\bar{x}}^2$, times the sample size, n. For each lot, the variance among the 50 values, $s_{\bar{x}}^2$, was computed as an estimate of $\sigma_{\bar{x}}^2$. Therefore

$$s^2 = ns_{\bar{x}}^2 \tag{5}$$

From equation 4

$$k = \frac{m^2}{s^2 - m} \tag{6}$$

Substituting equation 5 into equation 6, the moment estimate of k is

$$k = \frac{m^2}{ns_{\bar{x}}^2 - m} \tag{7}$$

Estimates of m, k, and $s_{\bar{x}}^2$ are computed from the 50 sample aflatoxin test results for each lot and for each sample size or a total of 120 estimates of m, k, and $s_{\bar{x}}^2$.

Comparison of Observed and Theoretical Distributions

Equation 2 defines the hypothesized theoretical family of negative binomial distributions for $\bar{X}$, denoted by $C(\bar{X})$ indexed by the parameters K and M. Using the same equation and the estimates of the parameter values, k and m, respectively, we calculated a fitted cumulative distribution, which we denote by $c(\bar{x})$. The Kolmogorov–Smirnov (KS) test (10) was used to test the null hypothesis H_0 that the observed cumulative distribution of $\bar{x}$ values, denoted by $F(\bar{x})$, could be the consequence of a random sample from a member of the family $C(\bar{X})$. The test is based on the greatest absolute differences D_{max} between $F(\bar{x})$ and $c(\bar{x})$. If D_{max} is greater than some critical value D_c, then the null hypothesis H_0 that $F(\bar{x})$ arises from a member of the $C(\bar{X})$ family is rejected with significance α. For a distribution with 50 sample aflatoxin test results, the D_c for 95% confidence limits is 0.188 (11).

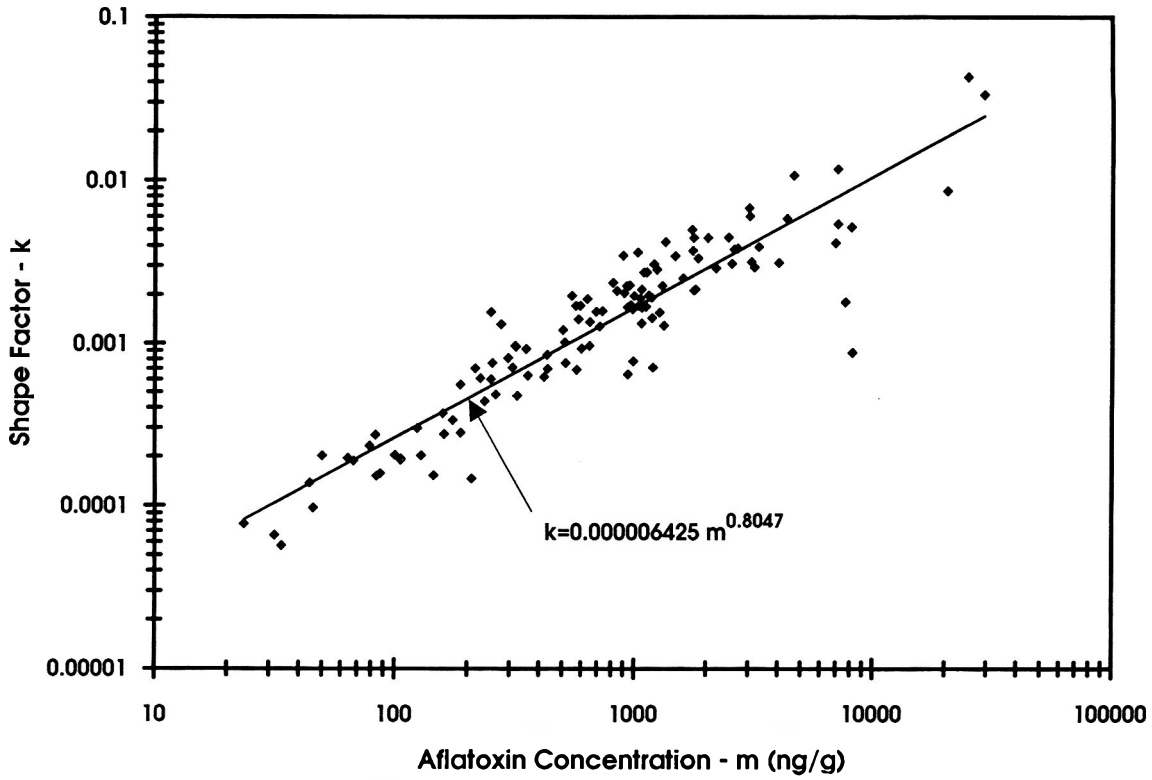

Figure 1. Relationship between the negative binomial shape parameter, *k*, and aflatoxin concentration, *m*, for 40 contaminated farmers' stock lots. Three estimates of *k* per lot are shown, using 2.26, 4.21, and 6.91 kg sample sizes. Coefficient of determination is 0.85 in the log scale.

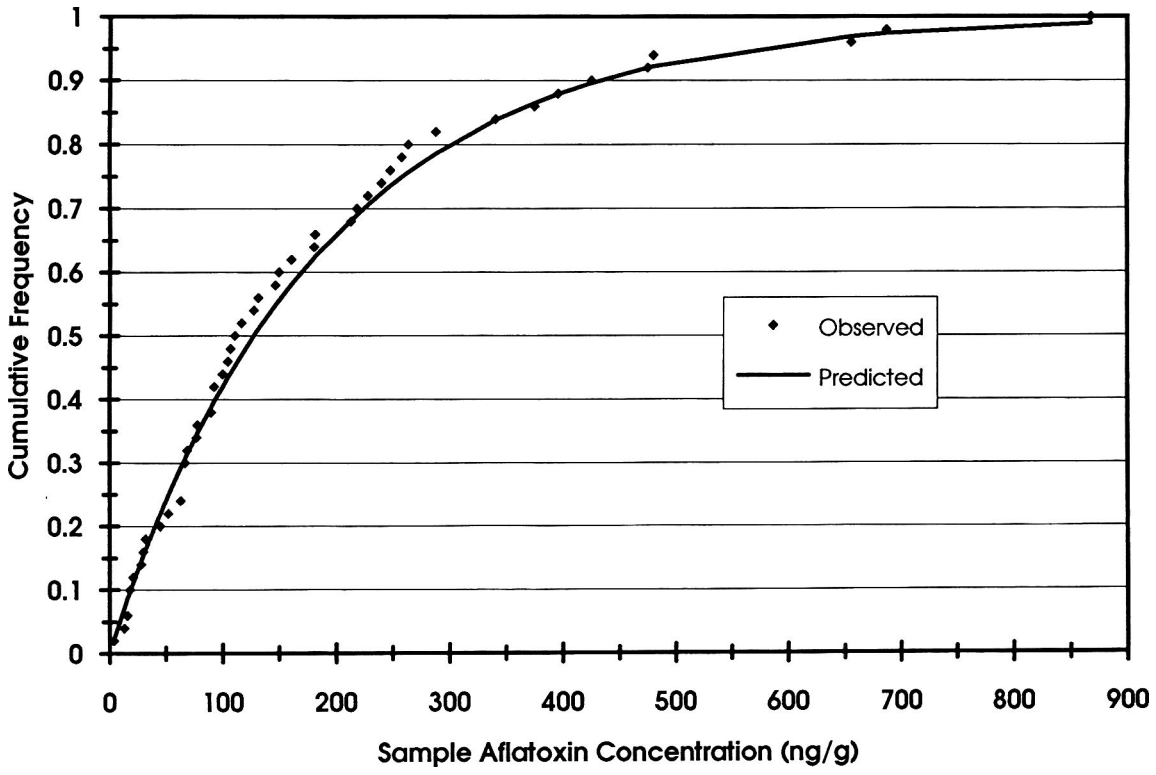

Figure 2. Comparison of the negative binomial function and the observed distribution of sample aflatoxin test results for lot 30 and the 2.26 kg sample size. The maximum difference between the observed and predicted distributions was 0.052, which occurred at a sample concentration of 63 ng/g.

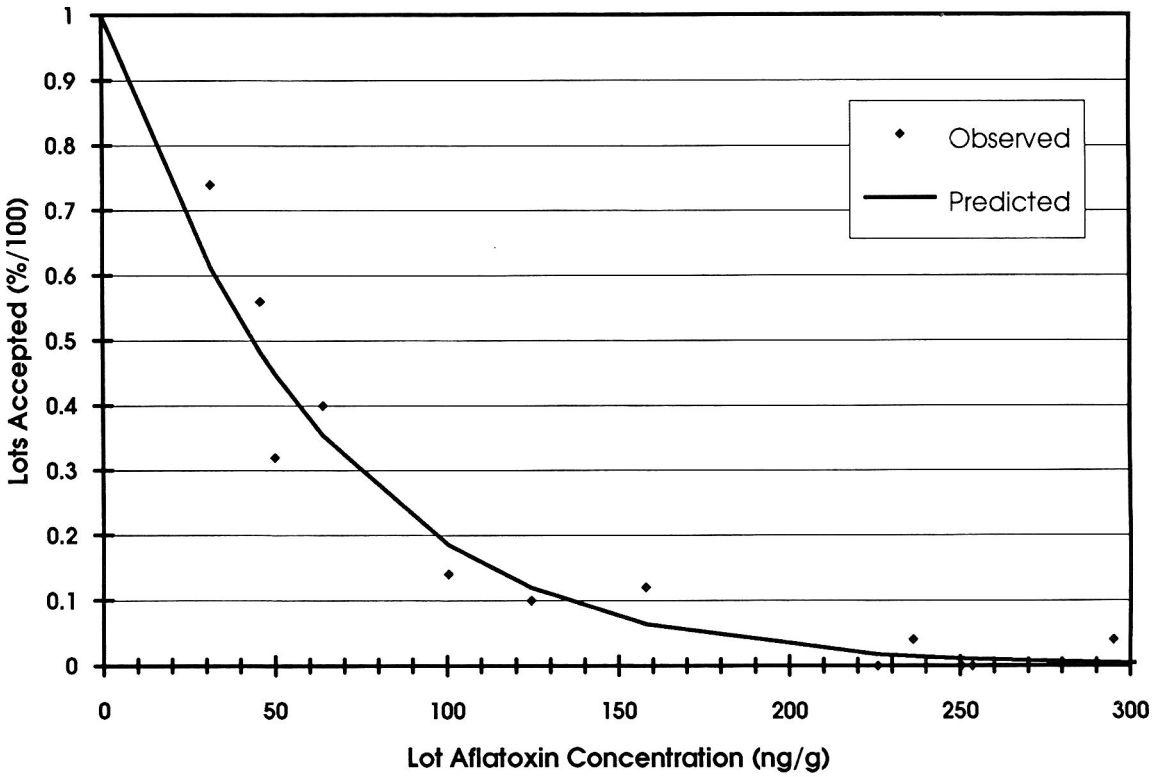

Figure 3. Observed and predicted acceptance probabilities for a 4.21 kg sample and a 20 ng/g sample acceptance level.

The KS test is exact when the hypothesized family of distributions is continuous and when the parameter values used to compute $c(\bar{x})$ are known in advance or at least estimated from an independent data set (10). The fact that the family of distributions used in this study is not continuous makes the test conservative (10). Little is known about the D_c statistic when the parameters are estimated from the observed data except that the critical value of D_c listed in the tables should be reduced slightly (10). However, Kendall and Stuart (12) indicate that if $F(\bar{x})$ lies completely within the band $c(\bar{x}) \pm D_c$, then the null hypothesis H_0 that we are sampling from a member of $C(\bar{X})$ cannot be rejected with significance α.

Results and Discussion

The average aflatoxin concentration, m; variance, $s_{\bar{x}}^2$; number of pods, n; and shape parameter, k, among the 50 sample test results for each sample sizes and for each lot are shown in Tables 1–3. The sample sizes averaged 2.26, 4.21, and 6.91 kg. The pod count averaged 884.5 pods per kg. The lots are ranked according to the average aflatoxin concentration, m, in Tables 1–3. For each sample size, the shape parameter, k, and the variance, $s_{\bar{x}}^2$, increase with lot aflatoxin concentration, m. Whitaker et al. (2) developed the relationship between the variance, s_x^2, and aflatoxin concentration, m, in a previous study. The variance among sample concentrations, $s_{\bar{x}}^2$, is always greater than the lot concentration, m. This implies that the variance among the pod concentrations, σ^2, is greater than M, which is a necessary condition for the negative binomial function to be applicable.

Because k reflects the distribution of pods according to their aflatoxin concentration in a contaminated lot (equation 1), the values of k should be independent of sample size. All 120 values of k are plotted versus m in a full $\log_e$ plot in Figure 1. The full log plot indicates that a power function of the general form

$$k = c1m^{c2} \tag{8}$$

where c1 and c2 are constants, describes the empirical relationship between the shape parameter, k, and aflatoxin concentration, m. By using the Statistical Analysis System (13), the following regression equation was obtained:

$$k = 0.000006425m^{0.8047} \tag{9}$$

with a coefficient of determination of 0.85 in the log scale. The standard error of estimate for (log c1) and c2 coefficients are 0.2085 and 0.0312, respectively.

Theoretical and Observed Distributions

By using the k, m, and n parameters in Tables 1–3, the negative binomial function was used to compute 120 fitted cumulative distributions of sample aflatoxin test results, $c(\bar{x})$. Each fitted cumulative distribution was then compared with each of the 120 observed cumulative distributions. An example of one such comparison is shown in Figure 2 for lot 30 and a 2.26 kg sample. The maximum difference, D_{max}, between the 2 distributions is 0.052 and occurs at a sample concentration or value of 63 ng/g. The remaining D_{max} values for each lot comparison

Table 4. Maximum difference, D_{max}, between the observed distribution and negative binomial distribution for each sample size and each farmers' stock lot[a]

Lot	Sample size, kg		
	2.26	4.21	6.91
1	0.1055	0.0724	0.0594
2	0.0976	0.0566	0.0672
3	0.1101	0.1189	0.0933
4	0.0600	0.1056	0.0781
5	0.0416	0.1409	0.0563
6	0.1345	0.0703	0.1200
7	0.0726	0.0894	0.0648
8	0.0568	0.0791	0.0815
9	0.1469	0.0879	0.0837
10	0.1042	0.0955	0.0660
11	0.0577	0.1115	0.1336
12	0.0507	0.0805	0.0902
13	0.0511	0.0612	0.0704
14	0.0812	0.0693	0.0672
15	0.0930	0.0681	0.0799
16	0.0614	0.0836	0.0500
17	0.0911	0.0946	0.0611
18	0.2144[a]	0.1800	0.0653
19	0.1602	0.0726	0.0751
20	0.0625	0.0552	0.0601
21	0.0620	0.0616	0.0922
22	0.0616	0.0694	0.1090
23	0.0891	0.0701	0.0888
24	0.0949	0.0883	0.0747
25	0.0850	0.0644	0.1337
26	0.0753	0.0681	0.0997
27	0.4292[a]	0.5627[a]	0.4200[a]
28	0.4658[a]	0.2300[a]	0.1199
29	0.0892	0.0878	0.1487
30	0.0524	0.0495	0.1085
31	0.0732	0.0899	0.0989
32	0.0812	0.0816	0.0715
33	0.0812	0.0608	0.0891
34	0.0667	0.1023	0.0904
35	0.0654	0.0677	0.1250
36	0.1280	0.0885	0.0788
37	0.0640	0.0997	0.0743
38	0.0746	0.0472	0.0729
39	0.1374	0.1194	0.1301
40	0.0623	0.0660	0.0659

[a] Values with an [a] indicate that $D_{max} > D_c$, where $D_c = 0.188$ for 50 degrees of freedom and 5% significance. D_{max} values $\leq D_c$ indicate that the true unknown distribution is equal to the negative binomial distribution and cannot be rejected at the 5% significance level.

is shown in Table 4. For the 120 distribution comparisons, D_{max} exceeded D_c for 6 lots. The null hypothesis, H_0, that the true unknown distribution is a member of the negative binomial distribution family cannot be rejected at the 5% significance level for 114 of the 120 distributions. Theory indicates that even if the negative binomial family was the true distribution, one would expect $D_{max} \geq D_c$ to occur 5% of the time or 6 times out of 120 fits, which is exactly what occurred in this study.

The negative binomial family of distributions failed the KS test 3 times out of 40 lots where the sample size was 2.26 kg, 2 times out of 40 lots where the sample size was 4.21 kg, and 1 time out of 40 lots where the sample size was 6.91 kg (Table 4). The condition where D_{max} exceeded D_c always occurred at $\bar{x}$ = 0 ng/g. These lots had the highest percentage of samples with no measurable aflatoxin. The negative binomial distribution predicted a lower percentage of sample aflatoxin test results of 0 than observed. This problem also occurred when fitting the negative binomial distribution to sample aflatoxin test results for shelled peanuts (4).

It appears that the poor fit ($D_{max} > D_c$) at 0 ng/g for farmers' stock peanuts is due to one or more of the following situations: (*1*) the limit of detection associated with the analytical and quantification method prevents a true 0 ng/g measurement (an observed 0 can be some positive concentration greater than 0 ng/g); (*2*) some other probability distribution function may fit the observed distribution of sample aflatoxin test results, specifically at $\bar{x}$ = 0 ng/g, better than the negative binomial family; and (*3*) some fitting procedure other than the method of moments may provide parameter estimates for the negative binomial distribution that gives better fits at $\bar{x}$ = 0 ng/g.

The negative binomial function assumes that an observed 0 is a true 0 and does not account for the limit of detection associated with the analytical method. For the analytical procedure used in this study, the limit of detection is estimated to be about 1 to 2 ng/g of total aflatoxin. For the 6 lots that failed the KS test, the fits were acceptable for sample concentrations greater than 0 ng/g in 4 of the 6 lots.

The fact that D_{max} exceeded D_c at 0 ng/g could also be due to the choice of the theoretical distribution. There are several skewed distributions other than the negative binomial (e.g., gamma, log normal, noncentral chi square) that may fit the observed distribution, specifically at 0 ng/g, more accurately (14–16). Future studies are planned to compare other theoretical distributions with the observed distribution of sample test results. For the negative binomial distribution, as well as for other theoretical distributions, there are usually more than one fitting technique used to determine the parameters of the distribution function. Perhaps other fitting techniques (e.g., maximum likelihood, percent zero) might provide better fits at 0 ng/g than the method of moments. Future studies will also investigate this effect.

Operating Characteristic Curves

As a consequence of an aflatoxin-sampling plan, a farmer's lot is judged acceptable or unacceptable depending on the analysis of samples drawn from the lot. A lot may be termed unacceptable when the sample aflatoxin test result $\bar{x}$ is above some predefined sample acceptance level $\bar{x}_a$. A lot is termed acceptable when $\bar{x} \leq \bar{x}_a$. Lots with an aflatoxin concentration M will be accepted as good with a certain probability $P(M) = (\bar{x} \leq \bar{x}_a \mid M)$. A plot of $P(M)$ versus M is called an operating characteristic (OC) curve. The probability of obtain-

ing a sample aflatoxin test result $\bar{x} \leq \bar{x}_c$ can be estimated from equation 2 with specified values of M, K, N, and $\bar{x}_a$.

Consider a sampling plan where a 4.21 kg sample is drawn from a lot, the entire sample is comminuted in a VCM, aflatoxin is extracted from a 100 g subsample, the aflatoxin is quantified by LC, and a sample acceptance level of 20 ng/g is used to classify lots. Figure 3 shows the OC curve associated with the above sampling plan. The acceptance probabilities were computed by using equation 2 for various lot concentrations, m; a sample size, n, of 3724 pods (pod count of 884.5 pods per kg); and k values determined from equation 9. The OC curve (Figure 3) for a 4.21 kg sample and a 20 ng/g sample acceptance level shows the percent lots at given lot concentrations accepted by the sampling plan. For example, a farmer's lot at 50 ng/g will be accepted about 45% of the time and rejected 55% of the time by the sampling plan. Lots with concentrations above 250 ng/g will be rejected about 100% of the time.

By using the 40 observed distributions of sample aflatoxin test results for the 4.21 kg sample size, up to 40 point estimates of acceptance probability, $p(m)$, can be determined and compared with the OC curve developed by equation 2. The observed acceptance probability for a given lot is the ratio of the number of sample aflatoxin test results that are 20 ng/g or less divided by the total number of sample aflatoxin test results. Each acceptance probability is plotted versus the lot concentration, m, and is also shown in Figure 3 along with the OC curve developed by equation 2 [observed acceptance probabilities where $p(m) = 0\%$ are not shown for m > 300 ng/g]. All $p(m)$ values, except the one for lot 24 where $m = 295$ ng/g, lie within the 95% confidence band of $P(m) \pm \Theta$ where

$$\Theta = \frac{\sqrt{P(m)[1 - P(m)]}}{\sqrt{40}} \qquad (10)$$

One should expect to get reasonably good fits between the observed and predicted OC curves because the negative binomial function provided good fits to each observed distribution of sample aflatoxin test results for $\bar{x}$ values greater than 0 ng/g. Because of the poor fit at $\bar{x} = 0$ ng/g, the negative binomial function along with the estimated parameters (determined by the method of moments procedure) will not accurately predict an OC curve for any sampling plan with a sample acceptance level of 0 ng/g. However, a sample acceptance level of 0 ng/g is not likely to occur in regulatory practice in the near future.

A study is required to compare various theoretical distributions and fitting techniques to the observed distributions described in this paper to determine if the fits, specifically at 0 ng/g, can be improved. However, if the poor fit at 0 ng/g is due only to the limit of detection associated with the analytical method, special fitting techniques will have to be applied regardless of the theoretical distribution used to describe the distribution of sample aflatoxin test results. The negative binomial function along with parameters determined by the method of moments can be used to develop an accurate statistical evaluation method to characterize proposed sampling plans for farmers' stock peanuts when using a sample acceptance level greater than 4 ng/g.

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FOOD COMPOSITION AND ADDITIVES

Determination of Polymerized Triglycerides in Frying Fats and Oils by Gel Permeation Chromatography: Interlaboratory Study

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An interlaboratory study of the gel permeation chromatographic (GPC) determination of polymerized triglycerides (PTG) in frying fats and oils was conducted by the Project Group on Collaborative Studies (PCS) of the Inspectorate for Health Protection, Food Inspection Service, The Netherlands. Thirteen laboratories participated in this study, which was focused on thermally abused oil and fat samples containing dimerized and polymerized triglycerides at levels just below and far above the Dutch regulatory limit of 16% (m/m). Samples were dissolved in tetrahydrofuran (THF) and analyzed on a GPC system, using THF as the mobile phase. Refractive index (RI) detection was used to determine PTG. Six heat-processed fat samples containing from 14 to 28% (m/m) PTG at 3 different levels (3 pairs of split level samples) were single analyzed according to the proposed procedure. Data analysis was performed according to the International Union of Pure and Applied Chemistry/International Organization for Standardization/AOAC protocols for statistics. The results of 2 collaborators were identified as outliers at the 20% (m/m) PTG level by applying the paired Grubbs test at the 1% level of significance. The repeatability relative standard deviation (RSD_r) values ranged from 0.48 to 2.25%, whereas the reproducibility relative standard deviation (RSD_R) values ranged from 1.34 to 4.57%.

In recent years, the production and consumption of fried foods (chicken, fish and chips, french fries, etc.) have increased considerably. During the frying process, oils and fats undergo chemical and physical changes and yield a variety of degradation compounds (1–4), including free fatty acids (FFA), soaps, volatile oxidation products such as aldehydes, ketones, acids, and various larger degradation products such as dimerized and polymerized triglycerides (PTG). Prolonged heating or overheating leads to high contents of these degradation products (1, 3, 5–7), giving fried foods an off-flavor and a bad taste. Moreover, such deteriorated fats and oils may be harmful to human health (3).

The regulations and analytical methods enforced by various countries to control the quality of heat-processed frying fats and oils in food establishments have recently been reviewed (8). The Dutch Food Act (9) permits up to 16% (m/m) PTG in frying fats and oils. Above this level, food processors are forced by law to replace their batch of frying fat or oil. Various analytical procedures have been described for the determination of PTG. A gas-liquid chromatographic method for the determination of polymers and oxidative products in heated vegetable oils was collaboratively studied (10) and adopted by AOAC INTERNATIONAL as an Official Method (11).

However, gel permeation chromatography (GPC) based on molecular exclusion chromatography combined with liquid chromatography (LC) is a more appropriate analytical procedure to determine PTG (including both thermal and oxidized polymers) in frying fats and oils (12–15). This method, which has a detection limit of 5% (m/m) PTG, was adopted by the International Union of Pure and Applied Chemistry (IUPAC) and the French and Dutch normalization organizations (16–18). The GPC procedure was tested in a collaborative study organized by IUPAC for PTG levels ranging from 2 to 10% (m/m). The results of this study have been published recently (19).

The 13 laboratories of the Dutch Food Inspection Services now use the GPC procedure (18, 19) to monitor the PTG content of used frying fats and oils. The application of this method at PTG levels just below and far above the Dutch regulatory

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limit of 16% (m/m) was collaboratively studied to determine validation parameters such as the within and between laboratory reproducibilities. The results are presented in this paper.

Interlaboratory Study

Study Design

The Project Group on Collaborative Studies (PCS), which organized this study, tested the method with 6 samples of frying vegetable fat at PTG levels of approximately 15, 20, and 28% (m/m). These 6 samples consisted of 3 pairs of split level samples (i.e., nearly identical materials that differ only slightly in analyte concentration).

Preliminary Study

The method instructions were sent to the laboratories well in advance of the study to let the analysts familiarize themselves thoroughly with the procedure. A few weeks prior to the study, the collaborators received a training sample of sunflower oil containing approximately 18% (m/m) PTG. The participants were requested to analyze the sample 8 times in succession starting from different test portions and then to send their results and comments on the method to the organizer of the study.

Sample Preparation

A batch of deteriorated frying fat containing approximately 28% (m/m) PTG was prepared by heating 600 g fresh frying vegetable fat in an open stainless steel frying pan for 17 h at 230°C.

The collaborative samples were prepared by mixing this fat with fresh fat yielding 6 sample batches with PTG contents ranging from 14 to 28% (m/m) (Table 1). Before shipment, the homogeneities of these samples were verified by taking 10 replicate test samples at random from each batch and analyzing them for their PTG content according to the prescribed procedure.

From each batch, 4 g portions were weighed into glass bottles and identified by code numbers. The 6 samples were mailed to each of the 13 participating laboratories.

Simultanuously, each laboratory received the procedure, an instruction form for participants, a sample delivery form, a reporting sheet (double), and an agreement-to-participate form. The laboratories were instructed to store the samples in the refrigerator at 4°C and to perform the analyses within 4 weeks after receipt of samples.

Each laboratory was asked to perform one analysis on each sample.

Table 1. Survey of PTG concentrations (in % m/m) of fat samples prepared from deteriorated frying fat and fresh fat by mass dilution.

	Mass portion of fat added, g		
Sample code	Deteriorated fat	Fresh fat	PTG, % m/m
A	64	56	15
B	88	32	20
C	120	0	28
D	60	60	14
E	82	38	19
F	113	7	26

Statistical Calculations

Statistical evaluation of the interlaboratory study data was carried out according to the IUPAC/ISO/AOAC protocol (20) for a "split-level" study as described by Pocklington (21, 22).

METHOD

A. Principle

Samples are dissolved in tetrahydrofuran (THF). Polymerized triglycerides are separated from other components in sample by GPC with THF as the mobile phase and quantitated by refractive index (RI) detector.

B. Apparatus

Trade names and sources are for user information only.

(a) *LC system.*—Sampler, high pressure pump, injection valve with a 20 µL loop, electronic integrator and recorder.

(b) *GPC column.*—Stainless steel, 30 cm × 7.5–7.8 mm id, packed with a spherical gel consisting of styrene-divinylbenzene copolymer (e.g., Ultrastyragel, or equivalent), 5–10 µm particle size, pore size 500 Å (meeting system suitability requirements *E*).

(c) *RI detector.*—Temperature controlled, with sensitivity of at least 1.10^{-4} of refractive index.

C. Reagents

Trade names and sources are for user information only.

(a) *THF.*—Analytical grade, nonstabilized. *Note*: Stabilized tetrahydrofuran may be used provided that the stabilizer does not interfere with the chromatographic separation. BHT (3,5-di-tert-butyl-4-hydroxytoluene) up to a concentration of 50 mg/L complies with this requirement.

(b) *Sodium sulfate.*—Anhydrous, reagent grade.

(c) *Frying oil standard.*—Containing 15–20% (m/m) PTG. Prepare this standard by heating refined soybean oil for 15–20 h at ca 200°C. If necessary, dilute afterward the heat-processed oil with fresh soybean oil to obtain the PTG content of interest.

(d) *Filter paper.*—Blue band (Schleicher & Schull, Dassel, Germany, is a suitable supplier)

D. Sample preparation

Gently melt solid and semisolid samples before analysis. Homogenize liquid samples. Weigh to the nearest 0.01 g ca 0.2–0.5 g homogenized sample into a glass-stoppered test tube or conical flask. Add 10.0 mL THF and swirl until a clear solution is obtained. Dry the solution over sodium sulfate for 5 min and filter through filter paper or equivalent filter with pore size between 0.5 and 1.0 µm.

E. Procedure

Start-Up

Adjust the RI detector to constant temperature, preferably a few degrees above ambient temperature.

Purge LC pump and injection valve with THF. Connect column to injection valve and detector and adjust the THF flow to 0.5–1 mL/min. Wash the whole system, including the reference cell of the detector, with THF. Switch on integrator and recorder. Let the system equilibrate until baseline is stable.

System Suitability Test

The following guide numbers for retention, resolution, and number of theoretical plates are recommended to obtain satisfactory results:

Retention.—Inject 20 µL of the frying oil standard solution. Adjust the attenuation of the recorder to yield appr 95% FSD for the triglycerides peak (see Figure 1, peak 3). Retention times should be ca 10 min for polymerized triglycerides, 11 min for dimerized triglycerides, 12 min for triglycerides, and 13 min for free fatty acids, depending on brand, age, and condition of column.

Resolution.—Use the frying oil standard solution to check the resolution (R) between triglycerides and dimerized triglycerides (peaks 3 and 2, respectively). Calculate the R-value as follows:

$$R = \frac{\Delta}{W}$$

where Δ is the distance, in mm, between the relative maxima of the triglycerides peak (peak 3) and the dimerized triglycerides peak (peak 2), and W is the width of the triglycerides peak at the baseline, in mm, measured between the points of intersection between tangent and baseline.

The recommended R-value is at least 1.0. If necessary, adjust the THF flow to obtain this resolution.

Number of theoretical plates.—Use the frying oil standard solution to check the number of theoretical plates (n) for triglycerides. Calculate the n-value as follows:

$$n = 16\left(\frac{d}{w}\right)^2$$

where d is the retention distance, in mm, between the start and the relative maxima of the triglycerides peak (peak 3), and w is the width of this peak at the baseline, measured between the points of intersection between tangent and baseline, in mm.

The recommended n-value is at least 6.000 plates.

F. Determination

Fill sample cups of sampler with sample and standard solutions in a sequence of 1 standard solution followed by 5 or 10 sample solutions. Set running and integration time to 15–20 min and start sampler. Chromatograph standard and samples at ambient temperature with THF as mobile phase and a flow rate between 0.5–1 mL/min. After last cup has been sampled, let system operate for 20 min.

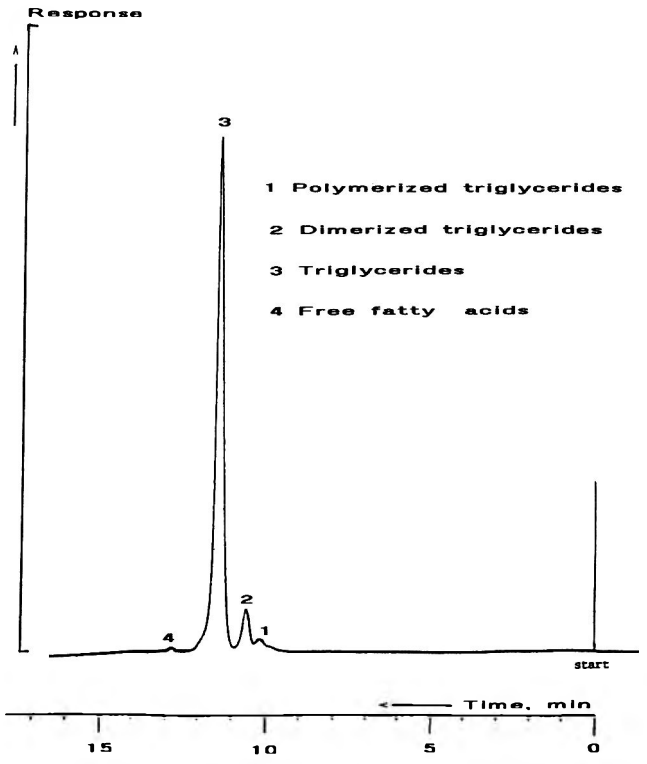

Figure 1. Chromatogram of a soybean oil with a PTG content of 12.3% (m/m) after heating fresh soybean oil for 18 h at approximately 180°C; Peak 1 = polymerized triglycerides (PTG); Peak 2 = dimerized triglycerides (DTG); Peak 3 = triglycerides (TG); Peak 4 = free fatty acids (FFA).

G. Calculation

Calculate PTG content of samples as follows:

$$PTG,\ \%\ m/m = \frac{A_p}{\sum A} \times 100$$

where A_p = the sum of the peak response values of the dimerized and polymerized triglycerides and ΣA the sum of all peak response values (including the fatty acid peak).

Report the results to the nearest 0.01% (m/m).

Results and Discussion

All data collected from the collaborators are presented in Table 2. The results of the 3 split-levels were paired for all laboratories and examined for outliers by the Cochran and Grubbs tests at the P = 1% level of significance. Collaborators 3 and 13 were identified as outliers as is shown in Table 2 at the 20% (m/m) PTG level by applying the paired Grubbs test for sample B and excluded from statistical evaluation. The results of Collaborator 13 are consistently lower than the other collaborators,

Table 2. Collaborative results for GPC determinations of PTG (in % m/m) in used frying fats

Collaborator	Split level 1		Split level 2		Split level 3	
	A	D	B	E	C	F
1	16.14	15.10	21.02	19.73	28.07	26.93
2	14.34	14.35	20.81	19.40	28.00	26.55
3	14.28	14.12	19.35[a]	19.02[a]	27.16	25.68
4	16.36	15.21	20.58	19.62	28.74	27.28
5	15.50	14.86	20.46	19.94	28.52	26.97
6	14.82	14.58	20.64	19.42	27.94	26.60
7	14.35	14.30	20.62	19.08	27.27	26.19
8	14.90	13.66	20.60	18.91	27.56	25.93
9	14.96	14.02	20.27	19.10	27.77	26.15
10	14.94	13.70	20.39	19.10	27.70	26.11
11	14.94	14.14	20.51	19.18	27.58	26.26
12	15.32	14.25	20.51	19.54	27.39	26.21
13	13.89	12.95	19.55[a]	18.38[a]	26.62	25.12
Mean	14.98	14.22	20.58	19.37	27.72	26.31

[a] Outliers by paired Grubbs tests (P = 1%)

but were not marked as stragglers or outliers by the outlier tests. The statistical results of this study are presented in Table 3.

The average PTG values for the split levels 1, 2, and 3 were 14.61, 19.97, and 27.01% (m/m), respectively. The repeatability relative standard deviations (RSD_r) for these levels ranged from 0.48 to 2.25% and are considered to be in agreement with this concentration range. The reproducibility relative standard deviation (RSD_R) reflecting the variation between the laboratories ranged from 1.34 to 4.57%. These values are acceptable according to the Harmonized IUPAC (1987) Protocol (20) and the Horwitz equation (23) dealing with the acceptability of collaborative studies representing a wide range of analytes, matrixes, and measurement techniques. According to these publications, the predicted RSD_R values for analyte concentrations between 14 and 29% should vary from 2.4 to 2.7%. According to the IUPAC (1989) Harmonized Protocols for Collaborative Studies (21), values within the range of 0.5 to 2 times the predicted RSD_R value may be considered as acceptable. For all samples analyzed, the repeatability values (r) varied from 0.37 to 0.92% (m/m), whereas the reproducibility values (R) ranged from 0.75 to 1.87% (m/m).

Table 3. Statistical analysis of the collaborative study GPC determination of PTG in used frying fats and oils

Parameter	Split level 1	Split level 2	Split level 3	Training sample
No. of laboratories	13	13	13	13
No. of results	26	26	26	104
No. accepted results	26	22	26	104
Mean, % m/m	14.61	19.97	27.01	18.08
s_r, % m/m[a]	0.33	0.22	0.13	0.20
RSD_r, %[a]	2.25	1.12	0.48	1.10
s_R, % m/m[a]	0.67	0.27	0.57	0.65
RSD_R, %[a]	4.57	1.34	2.11	3.60
r, % m/m[a]	0.92	0.63	0.37	0.56
R, % m/m[a]	1.87	0.75	1.59	1.84

[a] s_r and s_R are the repeatability and reproducibility standard deviations, respectively. RSD_r and RSD_R are the repeatability and reproducibility relative standard deviations, respectively. r and R are the repeatability ($s_r \times 2.8$) and reproducibility ($s_R \times 2.8$), respectively.

Results and statistical parameters obtained with the PTG training sample are also summarized in Table 3. These results do not significantly differ from those found in the collaborative study and, consequently, confirm the latter.

A representative GPC chromatogram of a heated soybean oil is shown in Figure 1. All collaborators used their own soybean oil standard with PTG contents ranging from 15 to 23% (m/m). Most of the chromatograms submitted by the collaborators for samples and standards were of good quality with regard to the peak shape of triglycerides and polymers.

The GPC system suitability recommendations for resolution ($R \geq 1.0$) and number of theoretical plates ($n \geq 6.000$) were not met in all cases. Collaborators 3, 4, and 9 reported n-values ranging from 3.700 to 4.400 plates, whereas Collaborator 9 found a resolution value of R = 0.6. Despite these findings, the results of these collaborators were not determined to be outliers (except for Collaborator 3 at the 20% (m/m) PTG level) and, therefore, accepted in this study. We concluded that the recommended values for n and R are indicative rather than compulsory.

The overall results obtained in this study demonstrate that GPC is a suitable procedure for the determination of PTG up to levels of 30% (m/m) in frying fats and oils. Furthermore, our findings confirm the results of other collaborative GPC studies for lower PTG contents (range: 2 to 22% [m/m]) in frying oils and fats as published by IUPAC and the French and Dutch normalization organizations (17–19).

Collaborators' Comments

In general, most collaborators commented favorably on the method. Only minor points were mentioned by the participants and no significant difficulties were experienced with the procedure.

Collaborators 4, 5, and 6 were not able to use thermostatically controlled refractive index detectors. The controlled temperatures reported by the other participants ranged from 27 to 40°C.

Collaborator 8 noticed that a negative peak with retention time of approximately 17 min was recorded on the chromatograms in the case of drying sample solutions with sodium sulfate. No negative peak was detected when sample solutions were not submitted to the drying process. The coordinator of this study noted that the occurrence of such a peak indicates that the THF in the reference cell of the RI detector is less dry than the THF used as mobile phase. The negative peak disappears when the reference cell is purged with mobile phase.

Recommendation

It is recommended that the gel permeation chromatographic method described in this report for the determination of PTG in heat-processed fats and oils be adopted as a validated method for the laboratories of the Food Inspection Services in The Netherlands.

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FOOD COMPOSITION AND ADDITIVES

Determination of *p*-Toluenesulfonamide in Ice Cream by Combination of Continuous Flow and Liquid Chromatography: Summary of Collaborative Study

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A collaborative study of the determination of *p*-toluenesulfonamide (*p*-TSA) in ice cream by a combination of continuous flow and on-line liquid chromatography was conducted. Seven ice cream samples containing 0–6.35 mg *p*-TSA/kg at 4 levels (1 blank and 3 pairs of split level samples) were analyzed by 11 laboratories. For all samples analyzed, the repeatability relative standard deviation varied from 2.08 to 3.67%, whereas the reproducibility relative standard deviation ranged from 7.79 to 11.68%. The average *p*-TSA values for the split levels 1, 2, and 3 were 0.55, 1.02, and 4.44 mg *p*-TSA/kg, respectively, with mean recoveries ranging from 76 to 79% (overall recovery range for all levels, 63–101%). No false positive results were reported for the blank sample, and no interference was encountered by the presence of vanillin in samples. The method has been adopted first action by AOAC INTERNATIONAL.

Chloramine-T (*N*-chloro-*N*-sodium-*p*-toluenesulfonamide) is commonly used in the food industry to disinfect equipment and machinery before processing. After addition, chloramine-T is converted into its reduction product *p*-toluenesulfonamide (*p*-TSA). A method based on a combination of continuous flow (CF) and on-line liquid chromatography (LC) to eliminate various laborious steps was applied to ice cream and subjected to collaborative testing. We previously presented a report of the collaborative study (*J. AOAC Int.* [1993] **76**, 570–574). The present report describes the method adopted first action by AOAC INTERNATIONAL.

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The recommendation was approved by the Committee on Commodity Foods and Products, and was adopted by the Official Methods Board of the Association. See "Changes in Official Methods of Analysis" (1994) *J. AOAC Int.* **77**, Jan/Feb issue, and "Official Methods Board Actions" (1993) *The Referee*, **17**, July issue.

993.22 *p*-Toluenesulfonamide Residues in Ice Cream—Combined Continuous Flow and Liquid Chromatographic Method

First Action 1993

(Applicable to determination of 0.5–5.0 mg *p*-toluenesulfonamide/kg ice cream.)

Method Performance:

Ice cream, 0.5 mg *p*-toluenesulfonamide/kg
Mean recovery = 78.8%
$s_r = 0.02$; $s_R = 0.04$; $RSD_r = 3.67\%$; $RSD_R = 7.79\%$
Ice cream, 1.0 mg *p*-toluenesulfonamide/kg
Mean recovery = 78.5%
$s_r = 0.02$; $s_R = 0.12$; $RSD_r = 2.08\%$; $RSD_R = 11.68\%$
Ice cream, 5.0 mg *p*-toluenesulfonamide/kg
Mean recovery = 76.1%
$s_r = 0.15$; $s_R = 0.46$; $RSD_r = 3.34\%$; $RSD_R = 10.30\%$

(*Caution*: *See Appendix: Laboratory Safety* for safe handling of organic solvents.)

A. Principle

Samples are homogenized and dialyzed to remove interferences (i.e., fats, proteins) in the continuous flow (CF) system. *p*-Toluenesulfonamide (*p*-TSA) is separated by on-line liquid chromatography (LC) and detected fluorometrically at 230 nm (excitation) and 295 nm (emission).

B. Apparatus

(**a**) *Gravimetric diluter.*—Capable of mass dilutions (1 + 4, m/m) (Spiral Systems Inc., Cincinnati, OH, is suitable source).

(**b**) *Homogenizer.*—Rotor/stator-type; do not use blender (Ultra Turrax, Janke & Kunkel, Staufen, Germany, and various models available from Fisher Scientific, Pittsburgh, PA, are suitable).

(**c**) *CF system.*—Equipped with sampler with external remote control of 6-port valve, dialyzer, tubing pump, *p*-TSA cartridge with 700 mm flat-plate dialyzer, and debubbler

(Skalar USA, Inc., Buford, GA, or Skalar Analytical BV, Breda, The Netherlands and Technicon Instruments Corp./Bran & Luebbe Analyzing Technologies, Inc., Elmsford, NY, or Maarssen, The Netherlands, are suitable sources). Connected to LC system, *see* Figure **993.22**. Operating conditions: sample time, 280 s; wash time, 270 s; airbubble time, 4 s. (*Note*: Flow time needed to transport sample from sampler to injection loop shall not be longer than washtime. Total sample and wash time of ≥550 s is necessary for LC separation.)

(**d**) *Valve.*—Automatic 6-port valve with 500 µL injection loop.

(**e**) *LC system.*—Equipped with high pressure pump, fluorescence detector capable of wavelength selections for excitation at 230 nm and emission at 295 nm, electronic integrator, and recorder. Operating conditions: LC mobile phase flow rate, 0.5 mL/min.

(**f**) *LC column.*—Reversed-phase C_{18}, 5 µm particle size, 3.0 mm id × 100 mm stainless steel (ss) to meet system suitability requirements in *E(1)* (Lichrospher or Lichrocart, E. Merck, Darmstadt, Germany, and Lichrosorb, Chrompack Int., BV, Middelburg, The Netherlands, are suitable sources).

(**g**) *Guard column.*—Reversed-phase C_{18}, 5 µm particle size, 3.0 mm id × 10 mm ss (Lichrosorb is suitable).

C. Reagents

(**a**) *Methanol.*

(**b**) *Phosphate buffer.*—pH 7.00, containing 3.522 g potassium dihydrogen phosphate (KH_2PO_4) and 7.265 g disodium hydrogen phosphate dihydrate ($Na_2HPO_42H_2O$) in 1 L H_2O (commercial buffer solutions are suitable).

(**c**) *CF buffer solution.*—Dissolve 2 g sodium chloride in 1 L phosphate buffer, (**b**).

(**d**) *Sampler wash solution.*—Add 1 mL polyoxyethylene lauryl ether (Brij 35, 30% v/v in H_2O, Atlas Chemical Ind., Wilmington, DE, and E. Merck are suitable sources) to 1 L H_2O. Mix.

(**e**) *LC mobile phase.*—Methanol–water (1 + 3). Dilute 250 mL methanol with 750 mL H_2O. Filter mobile phase through 0.45 µm filter.

(**f**) *p-TSA stock solution.*—1.000 mg *p*-TSA/L. Dissolve 100 mg *p*-TSA in 10 mL 95% (v/v) ethanol and dilute to 100 mL with H_2O. Solution is stable for at least 4 months at 4°.

(**g**) *p-TSA intermediate and standard solutions.*—*(1) Intermediate solution.*—Pipet 1 mL *p*-TSA stock solution, (**f**), into 100 mL volumetric flask and dilute to volume with H_2O. *(2) Standard solutions.*—0.1, 0.2, 0.3, 0.4, and 0.5 mg *p*-TSA/L. Pipet 1, 2, 3, 4, and 5 mL of intermediate solution into separate 100 mL volumetric flasks and dilute to volume with H_2O. Standard solutions are stable for at least 4 weeks at 4°.

(**h**) *Vanillin stock solution.*—1 mg/mL. Dissolve 100 mg vanillin (4-hydroxy-3-methoxy-benzaldehyde) in 100 mL H_2O. Solution is stable for 4 months at 4°.

(**i**) *Vanillin standard solution.*—20 mg/L. Pipet 1 mL vanillin stock solution, (**h**), into 50 mL volumetric flask and dilute to volume with H_2O. Prepare day of use.

D. Preparation of Test Solutions

Dilute melted ice cream samples (ca 5–10 g) 1:4 (by weight) with H_2O, using balance or gravimetric diluter, *B*(**a**). Homogenize [*B*(**b**)] mixture ca 1 min. Remove and discard upper, fat and/or foam, layer by suction.

E. Procedure

(1) Start-up.—Place all lines in their respective solutions. Let CF system equilibrate for ≥30 min. Equilibrate LC system until baseline is stable. To set fluorescence detector sensitivity, pump 0.1 mg *p*-TSA/L standard solution, *C*(**g**)*(2)*, through instrument and adjust recorder. Signal to noise ratio of 10:1 is recommended.

After system equilibration, determine resolution, *R*, between 0.1 mg *p*-TSA standard solution and vanillin standard solution, *C*(**i**). *R*, as calculated below, should be ≥5.0.

$$R = \frac{2(t_2 - t_1)}{W_1 + W_2}$$

where t_1 and t_2 are elution times of *p*-TSA and vanillin, respectively, and W_1 and W_2 are peak widths of vanillin and *p*-TSA, respectively.

Proceed when above requirements are fullfilled.

(2) Determination.—Fill sample cups of sampler in following order: 5 cups with 0.1–0.5 mg *p*-TSA/L standard solutions, *C*(**g**)*(2)*; 5 cups with test solutions, *D*; 1 cup with 0.3 mg *p*-TSA/L standard solution; 5 cups with test solutions; etc. Run H_2O sample at end of each series.

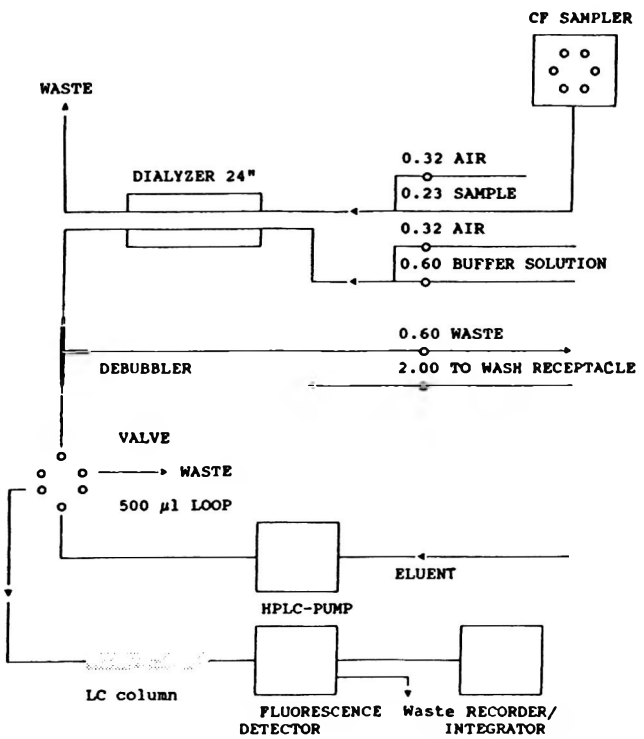

Figure 993.22. Flow diagram for combination of continuous flow and on-line liquid chromatographic system for determination of *p*-toluenesulfonamide in ice cream.

Start sampler. After last cup has been sampled, let system continue until steady baseline is obtained.

(3) Shut-down.—After each series of analyses, pump methanol–water solution (ca 80 + 20 v/v) through LC column until baseline is stable.

F. Calculation

Plot peak area vs concentration of *p*-TSA standard solutions. Determine *p*-TSA concentrations (C_x) of test solutions by interpolation. To calculate *p*-TSA concentration in samples, multiply C_x by 5 (dilution factor). Report results to nearest 0.01 mg *p*-TSA/1 kg sample.

Ref.: *JAOAC* **76**, 570-574 (1993)

FOOD COMPOSITION AND ADDITIVES

Determination of the Iodine Value of Oils and Fats: Summary of Collaborative Study

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Two collaborative studies were conducted using the Wijs method for determining the iodine value in a wide range of vegetable and animal oils and fats. The results obtained when using carbon tetrachloride were compared to those obtained when using a substitute solvent mixture of cyclohexane and glacial acetic acid. The values reported for the iodine values indicate that the cyclohexane and acetic acid mixture can be used in place of carbon tetrachloride without loss of precision. The method has been adopted first action by AOAC INTERNATIONAL as an IUPAC/AOCS/AOAC method.

Eleven laboratories from 9 countries participated in a collaborative study conducted in 1989. The test samples represented a range of lipid materials, including 7 vegetable oils consisting of olive oil, refined palm kernel oil, crude and refined palm oil, tung oil, sunflower seed oil, and hydrogenated soybean oil; 3 animal fats consisting of crude and hydrogenated fish oil; and tallow. Each of the 11 materials was provided as blind duplicates. The participants were required to determine the iodine value once only using carbon tetrachloride, and once only using a mixture of cyclohexane and glacial acetic acid (1 + 1).

Eighteen laboratories from 11 countries participated in a second collaborative study conducted in 1990 (1). The test samples included 3 materials in blind duplicates (hydrogenated soybean oils at 2 levels of hydrogenation, and hydrogenated fish oil). Solvents used in the second study were the same as those used in the 1989 study.

Submitted for publication December 12, 1992.

The recommendation was approved by the Committee on Food Nutrition and was adopted by the Official Methods Board of the Association. See "Changes in Official Methods of Analysis" (1994) *J. AOAC Int.* **77**, Jan/Feb issue, and "Official Methods Board Actions" (1993) *The Referee*, **17**, July issue.

D. Firestone is the AOAC INTERNATIONAL General Referee on Fats and Oils.

993.20 Iodine Value of Fats and Oils—Wijs (Cyclohexane–Acetic Acid Solvent) Method

IUPAC/AOCS/AOAC Method

First Action 1993

(Applicable to determination of iodine value for fats and oils that do not contain conjugated double bonds.)

Method Performance:

See Table **993.20A** for method performance data.

[*Caution*: Wijs solution causes severe burns; vapors can cause lung and eye damage. Use of fume hood is recommended. *See Appendix: Laboratory Safety* for procedures on safe handling of acids and organic solvents (cyclohexane).]

A. Principle

Fat or oil sample is mixed with iodine monochloride solution to halogenate double bonds in fat or oil. Excess iodine monochloride is reduced to free iodine in presence of potassium iodide, and free iodine is measured by titration with sodium thiosulfate using starch as indicator.

Table 993.20A Method performance for determination of iodine value by wijs method using carbon tetrachloride solvent or cyclohexane–acetic acid (1 + 1) solvent

	Mean value		s_r		s_R		RSD_r, %		RSD_R, %	
Sample	CTC[a]	CHX[b]	CTC	CHX	CTC	CHX	CTC	CHX	CTC	CHX
Sunflower	133.6	132.9	1.4	1.7	3.4	2.3	1.1	1.3	2.6	1.7
Refined palm	53.1	53.0	0.1	0.2	0.3	0.4	0.2	0.3	0.5	0.7
Crude fish	109.1	108.5	0.7	0.5	1.7	1.1	0.6	0.5	1.6	1.0
Tung	164.5	163.1	2.0	1.4	3.1	2.5	1.2	0.9	1.9	1.5
Tallow (beef)	47.2	46.9	0.2	0.2	0.5	0.4	0.5	0.5	1.0	0.8
Crude palm	52.5	52.6	0.3	0.4	0.4	0.5	0.5	0.8	0.8	1.0
Used frying	37.7	37.7	0.1	0.2	0.2	0.3	0.4	0.5	0.4	0.9
Palm kernel	18.2	18.3	0.01	0.01	0.03	0.04	0.1	0.1	0.2	0.2
Olive	82.3	82.2	0.2	0.5	0.6	0.8	0.3	0.6	0.7	0.9
HSBO[c]–1	102.6	102.3	0.5	0.8	1.8	1.9	0.5	0.8	1.7	1.8
HSBO–2	74.7	74.8	0.5	0.4	1.0	0.6	0.6	0.5	1.3	0.8
HFO[d]	73.0	72.8	0.4	0.4	0.7	0.6	0.6	0.6	0.9	0.8

[a] Carbon tetrachloride.
[b] Cyclohexane–acetic acid (1 + 1).
[c] Hydrogenated soybean oil.
[d] Hydrogenated fish oil.

Iodine value (IV), calculated as centigrams (cg) iodine absorbed per g sample (% iodine absorbed), is a measure of unsaturation of fats and oils.

B. Apparatus

(a) *Glass stoppered iodine flasks.*—500 mL.

(b) *Glass stoppered volumetric flasks.*—1000 mL, for preparing standard solutions.

(c) *Volumetric dispensers.*—*(1)* 25 mL, for Wijs and 15% potassium iodide (KI) solutions. *(2)* 2 mL, for starch solution. *(3)* 50 mL, for H_2O.

(d) *Repeater pipet.*—20 mL, with filling flask, for cyclohexane.

(e) *Analytical balance.*—Accurate to ±0.0001 g.

(f) *Filters.*—Ashless, coarse grade (Whatman No. 541 is suitable).

(g) *Hot air oven.*—Capable of maintaining 100° within ±1.5°.

C. Reagents

(a) *Potassium iodide (KI) solution.*—15%. Dissolve 15 g KI in 100 mL H_2O.

(b) *Wijs iodine solution.*—*(1)* Dissolve 13 g resublimed I in 1 L acetic acid, and pass in dried (through H_2SO_4) Cl until original $Na_2S_2O_3$ titration of solution is not quite doubled. (Characteristic color change at end point indicates proper amount of Cl. Convenient method is to reserve some of original I solution, add slight excess of Cl to bulk of solution, and bring to desired titer by readditions of reserved portion.) Or: (2) Dissolve 16.5 g ICl in 1 L acetic acid.

Determine I/Cl ratio as follows:

Iodine content.—Pipet 5 mL Wijs solution into 500 mL erlenmeyer flask containing 150 mL saturated Cl–H_2O and some glass beads. Shake, heat to boiling, and boil briskly 10 min. Cool, add 30 mL H_2SO_4 solution (1 + 49) and 15 mL 15% KI solution, and titrate immediately with 0.1N $Na_2S_2O_3$.

Total halogen content.—Pipet 20 mL Wijs solution into 500 mL erlenmeyer flask containing 150 mL recently boiled and cooled H_2O and 15 mL 15% KI solution. Titrate immediately with 0.1N $Na_2S_2O_3$.

$$\frac{I}{Cl} = \frac{2X}{3B - 2X}$$

where X = mL 0.1N $Na_2S_2O_3$ required for I content and B = mL required for total halogen content. If I/Cl ratio is not 1.10 ± 0.1, add I or Cl to correct ratio.

Standardized Wijs solution may be obtained from commercial suppliers (specify without carbon tetrachloride).

Store in amber bottle sealed with paraffin until ready for use. Wijs solutions are sensitive to temperature, moisture, and light. Store in dark at <30°.

(c) *Soluble starch solution.*—Mix paste of 1 g starch with small amount cold H_2O. While stirring, add 200 mL boiling H_2O. Test for sensitivity: place 5 mL starch solution in 100 mL H_2O and add 0.05 mL 0.1N iodine solution; deep blue color produced must be discharged by 0.05 mL 0.1N sodium thiosulfate solution. (*Note*: 1% starch solution, commercially available, is suitable.)

(d) *Potassium dichromate ($K_2Cr_2O_7$).*—Finely grind and dry to constant weight (ca 110°) before using in *D*.

(e) *Sodium thiosulfate ($Na_2S_2O_3 5H_2O$) solution.*—0.1N. Standardize as in *D*.

(f) *Acids.*—*(1) Hydrochloric acid (HCl).*—Concentrated, sp gr 1.19. *(2) Acetic acid ($C_2H_4O_2$).*—Glacial. *(3) Sulfuric acid (H_2SO_4).*—Concentrated.

(**g**) *Cyclohexane.*—(*Note*: Erratic results may result if cyclohexane is old, i.e., contains oxidizable matter; *see* (**h**).)

(**h**) *Cyclohexane–acetic acid solvent.*—Mix cyclohexane, (**g**), and acetic acid, (**f**)*(2)*, 1 + 1 (v/v). Verify absence of oxidizable matter in solvent by shaking 10 mL solvent with 1 mL saturated aqueous $K_2Cr_2O_7$ solution and 1 mL H_2SO_4, (**f**)*(3)*. No green color should appear.

D. Standardization of Sodium Thiosulfate Solution

Accurately weigh 0.16–0.22 g dried, finely ground $K_2Cr_2O_7$, *C*(**d**), to nearest 0.0001 g into 500 mL flask, dissolve in 25 mL H_2O, add 5 mL HCl, *C*(**f**)*(1)*, and 20 mL KI solution, *C*(**b**), and rotate to mix. Let stand 5 min.

Add 100 mL H_2O. Titrate with sodium thiosulfate solution, *C*(**e**), shaking continuously until yellow color has *almost* disappeared. Add 1–2 mL starch indicator solution, *C*(**c**), and continue adding thiosulfate solution slowly until blue color just disappears.

$$\text{Na}_2\text{S}_2\text{O}_3 \text{ solution normality, N} = \frac{20.394 \times \text{Wt } K_2Cr_2O_7}{\text{mL sodium thiosulfate}}$$

E. Determination

Melt test sample, if not already liquid (do not exceed sample melting point by >10°). Pass test sample through double layer of filter paper to remove any solid impurities and traces of H_2O (filtration may be performed in air oven, ca 100°, but should be completed within 5 ± 0.5 min). Sample must be absolutely dry. (*Note*: All glassware must be absolutely clean and completely dry.)

Let filtered test sample cool to 68–71°. Immediately weigh amount of test sample indicated in Table **993.20B** into clean, dry 500 mL flask, *B*(**a**).

Prepare at least 2 blank determinations to run with each sample group.

Add 15 mL cyclohexane–acetic acid solvent, *C*(**h**), to each test sample and swirl to ensure that sample is completely dissolved.

Dispense 25 mL Wijs solution into flask containing test sample, stopper flask, and swirl to mix. Immediately set timer for 1.0 or 2.0 h, depending on iodine value of sample (IV <150, 1.0 h; IV ≥150, 2.0 h) and store flasks in dark at 25 ± 5° for duration of reaction.

Table 993.20B Sample Weights

I value	Sample, g	Accuracy mg
3	10.58–8.46	± 0.5
10	3.17–2.54	± 0.2
20	1.59–1.27	± 0.2
40	0.79–0.63	± 0.2
80	0.40–0.32	± 0.2
120	0.26–0.21	± 0.2
160	0.20–0.16	± 0.2
200	0.16–0.13	± 0.2

Remove flasks from dark, add 20 mL KI solution, *C*(**b**), and mix. Add 150 mL H_2O and gradually titrate with 0.1N standard $Na_2S_2O_3$ solution, *D*, with constant and vigorous shaking or mechanical stirring. Continue titrating until yellow color has *almost* disappeared. Add 1–2 mL starch indicator solution to flasks and continue titrating until blue color has just disappeared. (*Note*: If reaction is not terminated by addition of KI and H_2O within 3 min past 1.0 or 2.0 h reaction time, sample must be discarded. The sample must be titrated within 30 min of reaction termination; if not, the analysis is invalid.)

F. Calculation

$$\text{Iodine value (IV)} = \frac{(B - S) \times N \times 12.69}{\text{Wt of sample}}$$

where B = titration of blank (mL); S = titration of sample (mL); N = normality of $Na_2S_2O_3$ solution.

Ref.: *Pure & Appl. Chem.* **62**, 2339(1990); JAOAC **77**, May/June 1994

Reference

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FOOD COMPOSITION AND ADDITIVES

Gas Chromatographic Determination of Mono- and Diglycerides in Fats and Oils: Summary of Collaborative Study

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A collaborative study was conducted on a capillary gas chromatographic method for the determination of mono- and diglycerides in fats and oils. Other components of fats and oils such as glycerol, fatty acids, and sterols may be analyzed by this method. Six materials used in the study consisted of 2 commercial mono- and diglyceride emulsifiers, 2 synthetic compositions with known amounts of mono- and diglycerides in the presence of an excess of triglycerides, and 2 refined sunflower oils spiked with mono- and diglycerides. Eight laboratories participated in the study. On the basis of the collaborative study results, the method has been adopted first action by AOAC INTERNATIONAL as an IUPAC/AOCS/AOAC method.

Mono- and diglycerides are natural constituents of oils and fats. They are also added as emulsifiers to oils and fats and used as food additives. A method was developed that involves conversion of the mono- and diglycerides into more volatile trimethylsilylether derivatives using a mixture (3 + 1) of *N,N*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS) in pyridine. Quantitation of mono- and diglycerides was by previously determined response factors to the internal standard *n*-tetradecane. Separation and detection was by capillary gas chromatography with split or on-column injection and flame ionization detection. Five laboratories applied split injection, and 3 laboratories applied on-column injection. Duplicate derivatives of the 6 materials were injected in duplicate (1).

Submitted for publication December 11, 1992.

The recommendation was approved by the Committee on Food Nutrition and was adopted by the Official Methods Board of the Association. See "Changes in Official Methods of Analysis" (1994) *J. AOAC Int.* **77**, Jan/Feb issue, and "Official Methods Board Actions" (1993) *The Referee*, **17**, July issue.

D. Firestone is the AOAC INTERNATIONAL General Referee on Fats and Oils.

993.18 Mono- and Diglycerides in Fats and Oils—Gas Chromatographic Method

IUPAC/AOCS/AOAC Method

First Action 1993

(Applicable to determination of mono- and diglycerides in concentrates and fats and oils. Other emulsifiers and components of fats and oils (glycerol, fatty acids, sterols, etc.) may be converted to trimethylsilylether derivatives and analyzed by this procedure.)

Method Performance:

See Tables **993.18A**, **993.18B**, and **993.18C** for method performance data.

(*Caution*: *See Appendix: Laboratory Safety* for safe handling of pyridine and hexane.)

A. Principle

Mono- and diglycerides are converted with *N,N*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS) in pyridine to volatile trimethylsilylether derivatives. Derivatives are separated by gas chromatography (GC) and detected by flame-ionization. *n*-Tetradecane is used as an internal standard.

B. Apparatus

(**a**) *GC system.*—Equipped with split injection or on-column injection, oven temperature programming, and flame-ionization detector. Operating conditions: split injection (split ratio 1:10–1:50); direct injection (splitless, hold for 1 min); injection port, 320° (or for on-column injection, 60°); column, initial, 80° (or for on-column, 60°); program rate, 10°/min; final temperature, 360°, hold 15 min; detector, 350°; carrier gas flow, 5 mL He/min (at 80°); injection volume, 1–5 μL. (*Note*: For on-column injection, or direct injection, dilute 50 μL reaction mixture with 1 mL hexane and inject 1 μL. When applying on-column injections, a precolumn may be used to lengthen column life. On-column injection gives more consistant response factors.)

(**b**) *Recording potentiometer and/or electronic integrator.*

(**c**) *GC column.*—0.25–0.35 mm id × 15–25 m glass or fused silica, surface fully deactivated by silylation agent, dimethylsilicone (SP-2100) or phenylmethyldimethylsilicone,

Table 993.18A Method performance for determination of mono- and diglycerides in 2 mono- and diglyceride concentrates (expressed as percent of mass of sample)

	Mean, %	s_r	s_R	RSD_r, %	RSD_R, %
1-Myristate	0.5	0.04	0.04	8.9	9.2
	1.7	0.05	0.1	3.0	5.7
1-Palmitate	17.1	0.7	1.9	4.1	10.9
	27.2	0.9	2.4	3.3	8.9
1-Stearate	23.6	0.8	3.3	3.4	14.1
	60.1	2.1	6.4	3.5	10.7
1,3-Dipalmitate-3-stearate	6.2	0.3	1.0	4.8	15.8
	0.2	0.01	0.06	4.5	30.0
1-Palmitate-3-stearate	17.4	0.6	4.1	3.4	24.2
	0.8	0.05	0.1	6.0	17.8
1,3-Distearate	13.0	0.4	3.0	3.1	23.2
	1.1	0.07	0.3	6.8	24.8

10% phenyl (OV-3) coating (or other phase with similar polarity), 0.1–0.2 μm film thickness. (*Note*: Use column length as required to separate mono- or diglycerides. Individual unsaturated mono- and diglycerides may not separate from saturated or less-unsaturated mono- or diglycerides. Thin-layer chromatography on silica gel impregnated with boric acid, immediately prior to derivitization, can be used to resolve glycerol-2-monoesters from glycerol-1-monoesters.)

(**d**) *Automatic sampler.*—Optional. (*Note:* For automatic samplers with 2 mL crimp-top vials, double sample and reagent amounts.)

(**e**) *Screw cap vials.*—2.5 mL (or 2.0 mL crimp-top vials for automatic sampler), with Teflon-faced septa.

(**f**) *Heating device for vials.*—Capable of maintaining 70 ± 0.5°.

C. Reagents

(**a**) *Silylating agents.*—*(1)* *N,N*-Bis(trimethylsilyl)trifluoroacetamide (BSTFA). *(2)* Trimethylchlorosilane (TMCS).

(**b**) *Pyridine.*—Store over KOH.

(**c**) *n-Hexane.*

(**d**) *Internal standard.*—*n*-Tetradecane, 99% min purity.

(**e**) *Internal standard solution.*—Accurately weigh ca 100 mg *n*-tetradecane, to nearest 0.2 mg, into 10 mL volumetric flask and dilute to volume with pyridine.

(**f**) *Reference standards.*—Glycerol; palmitic acid; 1-palmitoyl glycerol; 1-stearoyl glycerol; 1,2-dipalmitoyl glycerol; 1,3-dipalmitoyl glycerol; 1,2-distearoyl glycerol. All ≥99% purity (Nu Chek Prep, Inc., Elysian, MN, is suitable source).

(**g**) *Reference solutions.*—For each reference standard, accurately weigh, to nearest 0.2 mg, ca 100 mg reference standard, (**f**) and ca 100 mg *n*-tetradecane, (**d**), into 10 mL volumetric flask and dilute to volume with pyridine; or weigh ca 100 mg of mixture containing several (e.g., 5) reference standards, *each component being present in about same quantity*, and 100 mg *n*-tetradecane into 2 mL volumetric flask and dilute to volume with pyridine. (*Note*: 1 or more reference solutions can also be prepared without *n*-tetradecane. Silylation of reference solutions is then carried out as for sample solution, *D*(**a**), after addition of internal standard solution and silylating reagents.)

D. Determination

(**a**) *Sample solution.*—Accurately weigh, to nearest 0.2 mg, ca 10 mg homogenized emulsifier concentrates, or ca 50 mg oils and fats containing emulsifiers, into vial, *B*(**e**). Add 0.2 mL BSTFA, *C*(**a**)*(1)*, and 0.1 mL TMCS, *C*(**a**)*(2)*, and then 0.1 mL internal standard solution, *C*(**e**), to sample. Cap vial securely and shake vigorously. Heat reaction mixture 30 min in 70° heating device. Without delay, inject 1–5 μL reaction mixture into GC (previously equilibrated to stable base line). Carry out reaction 2×, duplicate injections per reaction.

(**b**) *Reference solution.*—Pipet 0.10 mL reference solution, *C*(**g**), into vial and add 0.2 mL BSTFA and 0.1 mL TMCS. Heat reaction mixture and inject into GC as in (**a**). Use concentration range of reference standards similar to range of components to be quantified in sample solution. Check linearity by plotting response factor vs concentration of reference solutions.

E. Identification of Mono- and Diglycerides

Analyze reference solution under same operating conditions as for sample solution. Identify peaks by comparison of

Table 993.18B Method performance for determination of mono- and diglycerides in 2 fortified oils (expressed as percent of mass of sample)

	Spike, %	Rec., %	Mean, %	s_r	s_R	RSD_r, %	RSD_R, %
1-Palmitate	1.00	0.9	96.0	0.03	0.12	3.3	12.0
	1.77	1.72	97.2	0.08	0.23	4.8	13.4
1-Stearate	1.00	0.9	98.0	0.03	0.14	3.4	13.8
	2.85	2.7	97.5	0.14	0.40	4.9	14.5
1,2-Dipalmitate	1.00	0.9	97.0	0.04	0.24	4.0	24.4
	2.06	1.9	96.1	0.06	0.53	2.8	26.9
1,3-Dipalmitate	1.00	0.9	93.0	0.02	0.19	2.5	20.2
1,2-Distearate	1.00	0.9	97.0	0.06	0.19	6.2	19.8
	0.68	0.7	112	0.06	0.20	8.0	26.2

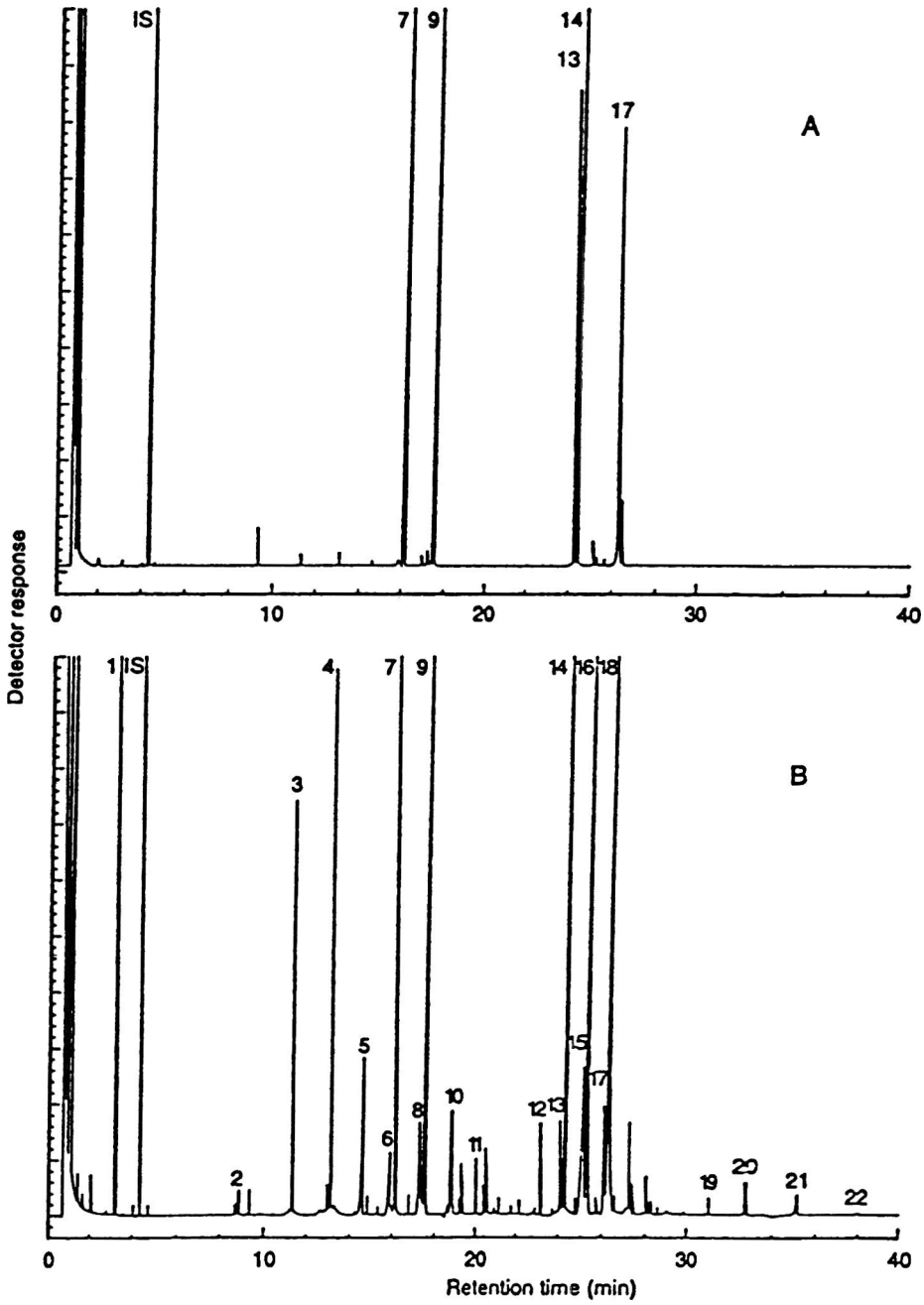

Figure 993.18.—Typical chromatograms of trimethylsilylether derivatives of mono- and diglycerides: A, reference standards and B, mono- and diglyceride emulsifier. The silylation procedure, column specifications, operating conditions, and peak identification are as follows:

(a) *Silylation.*—Sample size, 10 mg; reagents, 0.1 mL pyridine containing 1.0 mg *n*-tetradecane, 0.2 mL BSTFA, 0.1 mL TMCS; reaction time, 30 min at 70°.

(b) *Column.*—25 m × 0.31 mm id fused silica; 0.17 μm film thickness (5%phenylmethyl silicon, Ultra #2, Hewlett-Packard).

(c) *Operating conditions.*—Injector, 320°; hold 15 min; detector, 350°; carrier gas, He, 5 mL/min, 80°.

(d) *Peak identification.*—IS, *n*-tetradecane (internal standard); 1, glycerol; 2, diglycerol; 3, hexadecanoic acid; 4, octadecanoic acid; 5, glycerol 1-tetradecanoate; 6, glycerol 2-hexadecanoate; 7, glycerol 1-hexadecanoate; 8, glycerol 2-octadecanoate; 9, glycerol 1-octadecanoate; 10, glycerol 1-icosanoate; 11, glycerol 1-docosanoate; 12, glycerol 1-tetradecanoate-3-hexadecanoate; 13, glycerol 1,2-dihexadecanoate; 14, glycerol 1,3-dihexadecanoate; 15, glycerol 1-hexadecanoate-2-octadecanoate; 16, glycerol 1-hexadecanoate-3-octadecanoate; 17, glycerol 1,2-dioctadecanoate; 18, glycerol 1,3-dioctadecanoate; 19, triglyceride C48; 20, triglyceride C50; 21, triglyceride C52; and 22, triglyceride C54.

Table 993.18C Method performance for determination of mono- and diglycerides in blind duplicates of fortified sunflower oil (expressed as percent of mass of sample)

	Spike, %	Rec., %	Mean, %	s_r	s_R	RSD_r, %	RSD_R, %
1-Palmitate	0.80	0.7	91.9	0.07	0.14	10.0	18.5
1-Stearate	1.54	1.4	93.2	0.24	0.33	17.0	23.4
1,2-Dipalmitate	1.09	1.0	93.6	0.09	0.11	8.4	10.4
1,3-Dipalmitate	1.39	1.3	97.5	0.07	0.12	5.3	9.2
1,2-Distearate	2.18	2.3	114	0.30	0.94	11.8	36.3

retention time with known substances (or apply coupled GC/mass spectrometry). *See* Figure **993.18**.

F. Calculation

(**a**) *Response factor.*—Using reference solution chromatogram, calculate response factor, R_x, of reference standard vs internal standard.

$$R_x = \frac{m_{is}}{m_x} \times \frac{A_x}{A_{is}}$$

where R_x = response factor of reference standard x; m_{is} = mg internal standard; m_x = mg reference standard x; A_x = peak area of reference standard x; and A_{is} = peak area of internal standard.

Check response factors periodically. Response factors should be >0.5. Lower response factors indicate some loss or decomposition. Use 0.5–10 mg/mL components in both reference and sample solutions.

(**b**) *Calculation of sample component content.*—Calculate content of sample component x, m'_x (in mg %) as follows:

$$m_x' = \frac{1}{R_x} \times \frac{m_{is}'}{m_s'} \times \frac{A_x'}{A_{is}'} \times 100\%$$

where m_x' = mg % component x in sample; R_x = response factor of component x in sample; m_{is}' = mg internal standard in sample; m_s' = mg sample; A_x' = peak area of component x in sample; and A_{is}' = peak area of internal standard in sample.

Ref.: *Pure Appl. Chem.* **63**, 1153(1991). JAOAC **77**, May/June (1994).

Reference

(1) Brüschweiler, H., & Dieffenbacher, A. (1991) *Pure Appl. Chem.* **63**, 1153–1158

FOOD COMPOSITION AND ADDITIVES

Effect of Various Commercially Available Enzymes in the Liquid Chromatographic Determination with External Standardization of Thiamine and Riboflavin in Foods

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The efficacy of various commercially available enzymes in the determination of thiamine and riboflavin in foods was studied by liquid chromatography (LC) using external standards. Different enzymes, as well as the same enzyme produced by different manufacturers, very strongly affected the determination of both vitamins. The recoveries for different foods ranged from 85 to over 100% for thiamine and from 80 to 100% for riboflavin. The present LC method was accurate and precise when tested on a food and a feed reference material, and coefficients of variation were 5.5% for thiamine and 10% for riboflavin in a rye flour reference material tested for 8 months.

Several methods based on different principles are available for the determination of thiamine and riboflavin in foods. These include microbiological assays, fluorometric determination, and liquid chromatography (LC) coupled with fluorometric detection (1). Although the microbiological assay is highly reliable, it is tedious and time-consuming (1). Fluorometric methods are not very specific without LC separation of the vitamins (2). Therefore, LC-based methods have gained more acceptance in recent years (3). Most of the LC methods for the determination of thiamine and riboflavin in foods are based on the same basic principle, with modifications only in the extraction, cleanup, and chromatographic conditions. Lately, LC methods were developed that allow determination of both thiamine and riboflavin by simultaneous chromatographic separation (2–9).

The use of different commercial enzyme preparations has been problematic, because the variable efficacy of different enzymes used for hydrolysis results in highly different values (9). Even though there is only 1 published report on this particular subject, the problem may have general importance. Previously (9), takadiastase was used with another enzyme; clara-diastase (Fluka), which according to my experience gives excellent results, was not used. Erroneous results also may result from the use of different standards. For thiamine, the final result varies depending on the standard used.

The first goal of the present study was to establish a method that permits simultaneous extraction and cleanup for both vitamins in different foods by using similar chromatographic conditions. The second goal was to examine whether the external standard curve could be used for quantitative analysis. The third goal was to validate the method by determining the most efficient enzyme for hydrolysis. The fourth goal was to check which thiamine standard would give correct results.

To test accuracy and precision, various food reference materials (RM) prepared in my laboratory were analyzed. The method also was validated by analyzing samples used for interlaboratory comparison studies on the determination of the 2 vitamins in feeds.

Materials and Methods

Sample Preparation

The samples were prepared according to a modification of the method of Reyes et al. (7). About 5 g of finely ground or homogenized sample was weighed in duplicate into 125 mL Erlenmeyer flasks. Rye flour RM (6 g) was measured into another flask. This rye flour RM was routinely used in every sample series. Thirty milliliters of 0.1N HCl was added to all flasks, and the mixtures were then stirred. The samples were autoclaved at 125°C for 15 min. After the samples cooled to room temperature, the pH was adjusted to 4.0–4.5 with 2N sodium acetate. Five milliliters of the enzyme to be tested (clara-diastase from Fluka Co. was preferred) (*see Results and Discussion*) was added to all flasks, after which the flasks were incubated at 50°C for 3 h. Thereafter, 1 mL of 50% trichloroacetic acid (Riedel de Haën) was added, and the flasks were incubated for 15 min at 90°C. When the samples cooled to room temperature, the pH was adjusted to 3.5 with 2N sodium acetate, the sample volume was brought to 50 mL with distilled water, and the mixture was filtered through Whatman No. 42 filter paper. Five milliliters of 1% potassium ferricyanide (oxidizing agent) in 15% NaOH was added to 10 mL of the filtered extract. Phosphoric acid (0.5 mL) was added immediately, and the neutralized oxidized extract was passed through an activated C_{18} Sep-pak cartridge (Waters Associates). Interfering substances were

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removed with 2 mL of phosphate buffer and 2 mL of 5% methanol in phosphate buffer. The vitamin derivatives were eluted with 4.5 mL 50% methanol; the eluate was diluted to 5 mL with 50% methanol and then filtered through a 0.45 μm filter into a dark ampule for LC analysis. The preparations were carried out in a dimly lit room. The standards were treated similarly.

Standards, Enzymes, and Reference Materials

Both thiamine mononitrate (Hoffman-LaRoche; a gift from Roche, Inc.) and thiamine hydrochloride (Merck No. 500923) were tested as thiamine standards. Riboflavin (Hoffman-LaRoche; a gift from Roche, Inc.) was used for riboflavin. Stock solutions of thiamine mononitrate and thiamine hydrochloride, which contained 100 μg/mL in 20% ethyl alcohol adjusted to pH 3.5–4.3 with HCl, and of riboflavin, which contained 100 μg/mL in 0.02M acetic acid, were prepared separately and stored in darkness in a refrigerator. Combined working standard solutions (10 μg/mL of each) were prepared daily. The external standard curve ranged from 10 to 50 μg. To test the accuracy and precision of the method, as well as for long-term analytical quality control, our own rye flour RM was used. The efficacy of the various enzymes was tested by using an interlaboratory comparison sample coded MF 90 (mixed feed) supplied by the Office of the International Analytical Group (IAG), Oosterberk, the Netherlands. The sample had been analyzed for these vitamins by various Western European official agricultural laboratories.

Five different enzymes were tested. Tests without enzymes also were conducted. The following enzyme preparations were used: takadiastase (Serva No. 35740 and Fluka No. 86250), papain (Fluka No. 76222), α-amylase (Sigma No. A-0273), and clara-diastase (Fluka No. 27540).

LC Determination of Thiamine and Riboflavin

The LC instrumentation consisted of an HP1090 M high-performance liquid chromatograph (Hewlett-Packard) and an HP1046 A fluorometric detector (Hewlett-Packard). Separation by reversed-phase chromatography was accomplished with a 100 × 8 mm Bondapak C_{18} radial-pak cartridge in an RCM 8 × 10 radial-pak cartridge holder (Waters Associates). Separate chromatographic runs were performed for the determination of each vitamin. Thiamine (as thiochrome) was measured at an emission wavelength of 425 nm and an excitation wavelength of 360 nm. Riboflavin was determined at an emission wavelength of 520 and an excitation wavelength of 440 nm. The injection volume was 20 μL, and external calibration was used. The mobile phase was 35% methanol and 65% phosphate buffer (0.005M, pH 7.0) at a flow rate of 0.8 mL/min. The oven temperature was 30°C.

For the recovery tests, known volumes of the standard solutions (containing 10–15 μg vitamin) were added to the sample solution before the autoclaving step.

Table 1. Calibration data for thiamine[a]

Retention time, min	Peak No.	Signal description	Amount, μg	Level	Area
16.177	1	X	11.780	1	738.85
			23.560	2	1495.2
			35.340	3	2319.7
			47.120	4	3023.5

[a] $r^2 = 1.000$; linear regression = 64.753 (amount) − 10.120.

Results and Discussion

Tables 1 and 2 show the calibration data for thiamine hydrochloride as free thiamine and for riboflavin, respectively. The standard curve is automatically drawn through the origin. For preparation of standard curve, 1–6 mL of intermediate standard was used. The repeatability of the standard curve was tested over several days and found to be extremely good. Coefficients of variation (CV) for the slope were 2.7% for the thiamine standard curve and 3.2% for the riboflavin standard curve (n = 4). Results were adequately repeatable for the thiamine standard curve and were linear to 50 μg. The standard curve for riboflavin was linear to 40 μg. Figures 1a and 1b show chromatograms of thiamine and riboflavin standards, respectively.

Thiamine mononitrate was also tested as a standard, but the amount of sample was 75–89% smaller than with thiamine hydrochloride (Table 3). Thiamine mononitrate is susceptible to decomposition in aqueous solution, particularly in the presence of alkali and heavy metals such as iron and copper. This instability is one possible reason why the sample amount was so much smaller when using thiamine mononitrate as the standard compared with thiamine hydrochloride. In the routine procedure, thiamine hydrochloride was used as the standard, and the results were reported as free thiamine.

The efficacy of different enzymes was tested by using an interlaboratory comparison sample coded MF 90 (mixed feed) supplied by the IAG. The sample had been analyzed for these vitamins by various Western European official agricultural laboratories. The determinations were carried out during the winter of 1990–1991; the results were constant throughout that period. The mean ± standard deviation values obtained by the participating Western European official agricultural laboratories for the interlaboratory comparison sample were 4.94 ± 1.48 mg/kg (n = 7) for thiamine and 8.74 ± 2.35 mg/kg (n = 10)

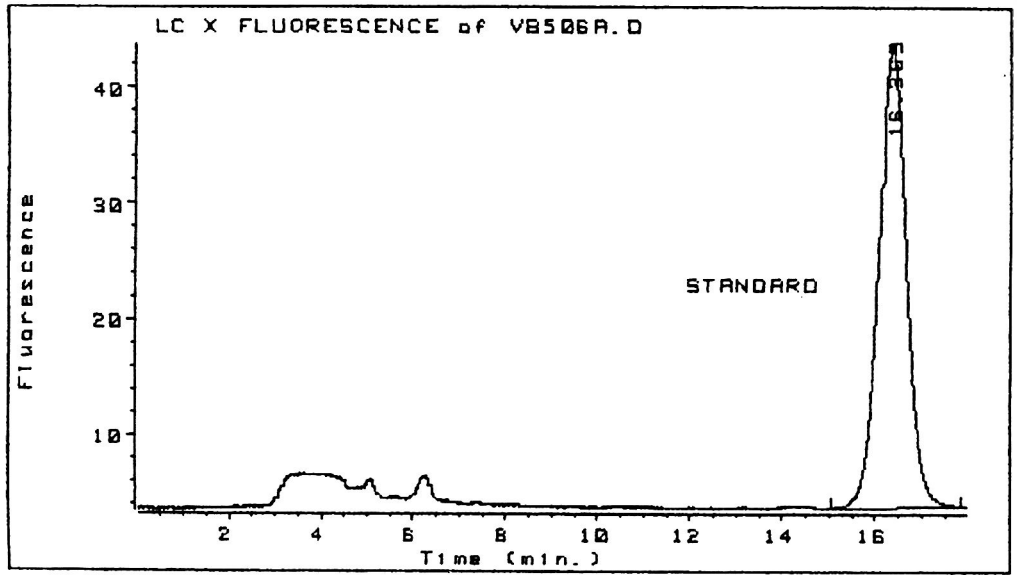
Table 2. Calibration data for riboflavin[a]

Retention time, min	Peak No.	Signal description	Amount, μg	Level	Area
14.734	1	X	10.050	1	261.28
			20.100	2	498.46
			30.150	3	737.36
			40.200	4	990.94

[a] $r^2 = 1.000$; linear regression = 24.457 (amount) + 6.016.

LC X FLUORESCENCE of VB506A.D
DATA:VB506A.D

Peak#	Ret Time	Type	Width	Area	Start Time	End Time
1	16.369	BBA	0.646	1668	15.060	17.867



LC X FLUORESCENCE of VB506B.D
DATA:VB506B.D

Peak#	Ret Time	Type	Width	Area	Start Time	End Time
1	17.000	BV	0.686	561.37	15.807	18.684

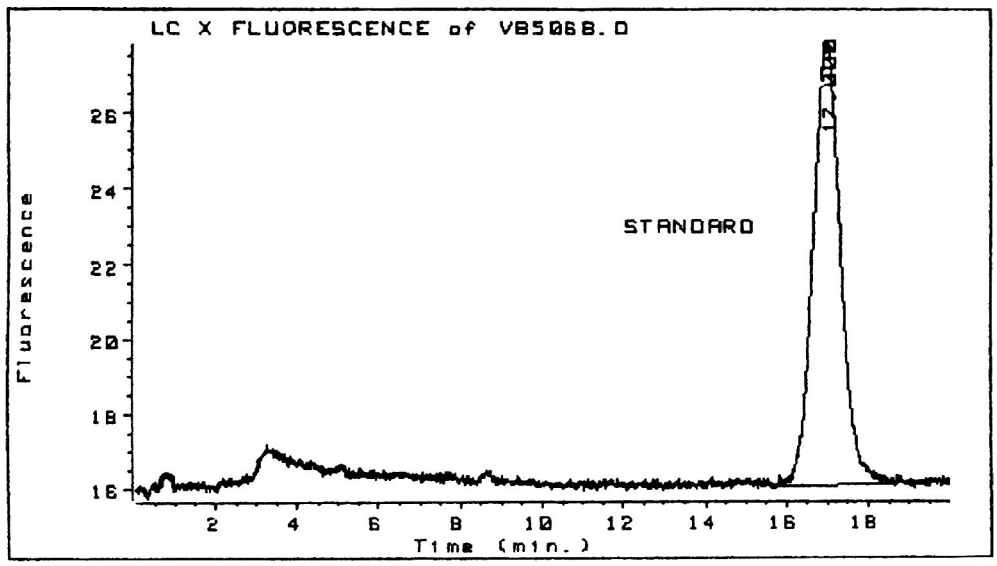


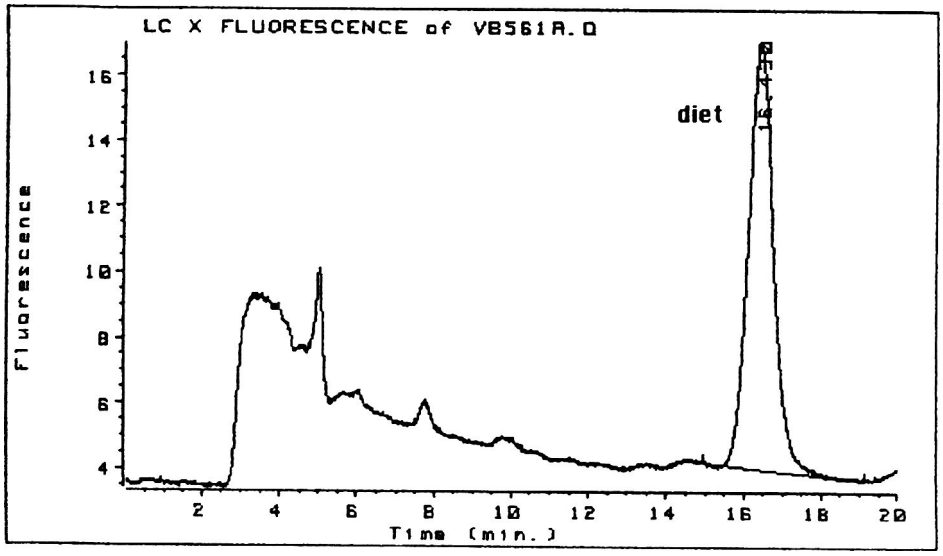
Figure 1. (a) Chromatogram of thiamine hydrochloride standard (21.24 μg; emission, 425 nm), and (b) chromatogram of riboflavin standard (23.26 μg; emission, 520 nm).

for riboflavin. In the present study, thiamine hydrochloride was used as the standard, which was prepared with the same enzyme (or without an enzyme) used with the sample. Table 4 presents the results for the interlaboratory comparison sample analyzed with various commercially available enzymes.

The different enzymes caused significant differences in the results. For thiamine determinations, results with 6% Fluka clara-diastase were 38% higher compared with those obtained with 6% Fluka takadiastase. For riboflavin determinations, 6% Fluka clara-diastase gave results that were 34% higher com-

LC X FLUORESCENCE of VB561A.D
DATA:VB561A.D

Peak#	Ret Time	Type	Width	Area	Start Time	End Time
1	16.430	BV	0.665	582.65	14.953	19.140



LC X FLUORESCENCE of VB562B.D
DATA:VB562B.D

Peak#	Ret Time	Type	Width	Area	Start Time	End Time
1	15.956	BV	0.615	234.12	14.960	17.019

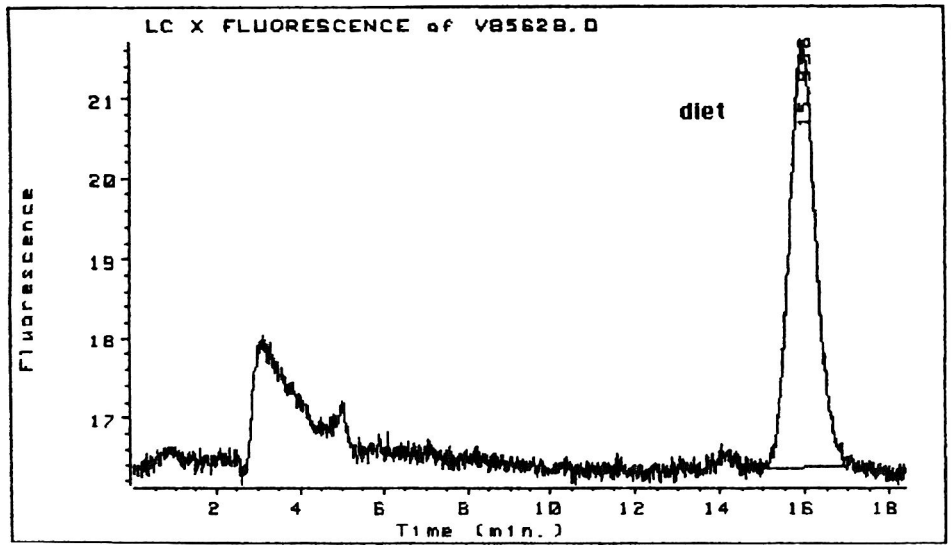


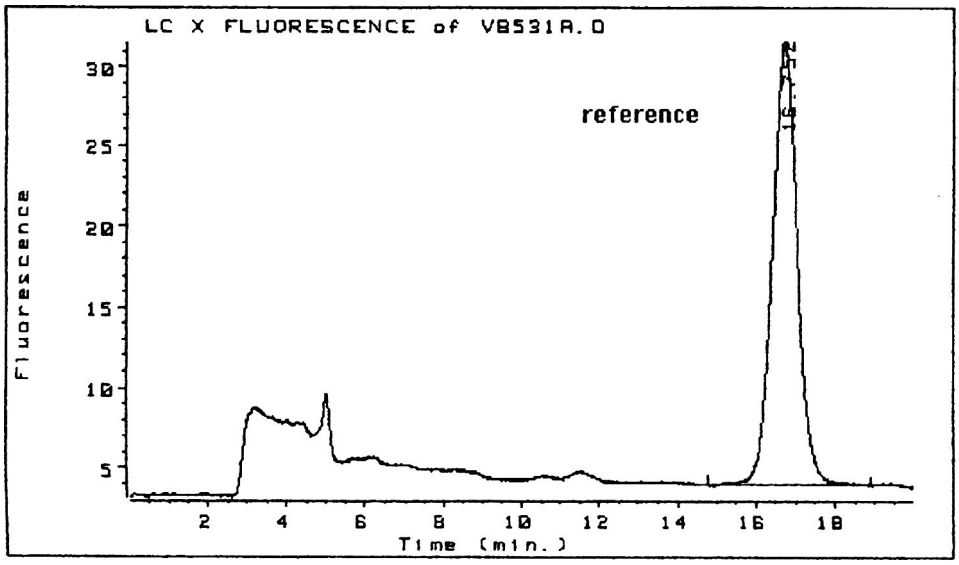
Figure 2. Chromatograms of (a) thiamine and (b) riboflavin determinations of a diet sample.

pared with those obtained with 6% Serva takadiastase. For thiamine determinations, papain gave a result that was 66% of that obtained with clara-diastase. For riboflavin, the difference between papain and clara-diastase was 71%. There was also a difference between Fluka takadiastase and Serva takadiastase for both riboflavin and thiamine. With Fluka clara-diastase, the results fell within 1 standard deviation of the mean value obtained by the laboratories participating in the interlaboratory comparison study. On the basis of these results, 6% Fluka clara-diastase was used in later work.

The recovery was also tested for different foods (Table 5). For crisp bread, the recovery depended on the particular type

LC X FLUORESCENCE of VB531A.D
DATA:VB531A.D

Peak#	Ret Time	Type	Width	Area	Start Time	End Time
1	16.752	BV	0.678	1206	14.767	18.873



LC X FLUORESCENCE of VB550B.D
DATA:VB550B.D

Peak#	Ret Time	Type	Width	Area	Start Time	End Time
1	14.905	VV	0.560	161.75	14.066	15.920

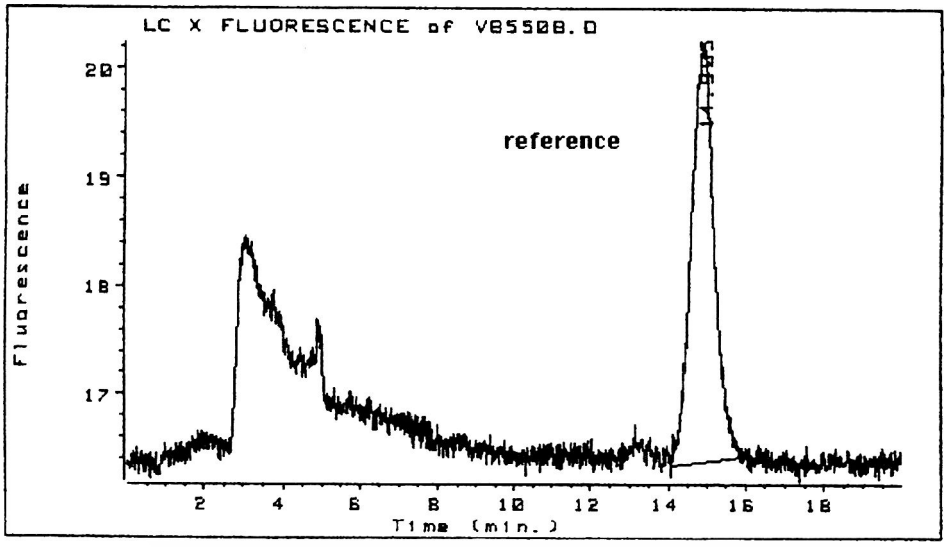


Figure 3. Chromatograms of (a) thiamine and (b) riboflavin determinations of our rye flour reference material.

of crisp bread. The recovery varied considerably, from 85 to 94% for thiamine and from 80 to 96% for riboflavin. For other foods, there were either minor or no differences in the recovery of thiamine. Recoveries for potatoes also varied, depending on the variety, particularly for riboflavin determinations. Figures 2a and 2b show the thiamine and riboflavin chromatograms for a diet sample.

The precision was tested with a rye flour RM prepared by our laboratory. A mean value of 2.73 μg/g was obtained for thiamine, with a standard deviation of 0.15 (CV, 5.5%) over an

Table 3. Effect of different standards on thiamine determination[a]

Sample	n	Standard 1[b]	Standard 2[c]	Ratio, %[d]
Milk	2	0.03	0.04	75
MF 90	2	2.7	3.6	75
Crisp bread	2	0.15	0.20	75
Crisp bread	2	0.20	0.23	87
Crisp bread	2	0.17	0.19	89

[a] Thiamine was determined as milligrams of free thiamine per 100 g of fresh weight.
[b] Thiamine mononitrate.
[c] Thiamine hydrochloride.
[d] Ratio, % = (thiamine found by using thiamine mononitrate standard)/(thiamine found by using thiamine hydrochloride standard) × 100.

8-month period (n = 60 determinations). For riboflavin, an average of 1.00 μg/g was obtained, with a standard deviation of 0.10 (CV = 10%) during the same interval (n = 54 determinations). Figures 3a and 3b show, respectively, a thiamine chromatogram and a riboflavin chromatogram for our rye flour RM. As shown earlier, the values obtained for thiamine and riboflavin depended on the enzyme used. For us, the Fluka clara-diastase was the best enzyme. Unfortunately, an earlier investigation reported that its availability is limited. Furthermore, the selection of the standard affects results.

Table 4. Effect of different commercially available enzyme preparations on apparent thiamine and riboflavin contents of an International Analytical Group MF 90 coded mixed feed reference material on a dry weight (dw) basis[a]

Enzyme	Thiamine, mg/kg dw	Riboflavin, mg/kg dw
Serva takadiastase, 6%	3.95 ± 0.52	6.79 ± 0.49
Fluka takadiastase, 6%	3.85 ± 0.39	7.48 ± 0.29
Fluka papain, 6%	3.49 ± 0.14	6.45 ± 0.30
Fluka clara-diastase, 6%	5.30 ± 0.11	9.09 ± 0.42
Sigma α-amylase, 6%	3.65 ± 0.29	7.32 ± 1.09
Fluka takadiastase, 3%, and Fluka clara-diastase, 3%	4.23 ± 0.02	7.94 ± 0.24
No enzyme	3.26 ± 0.03	5.80 ± 0.32

[a] Values are mean ± standard deviation; n = 4.

Table 5. Recoveries[a] of thiamine and riboflavin obtained for various foods with Fluka 6% clara-diastase

		Recovery, %	
Food	n	Thiamine	Riboflavin
Bread	4	100 ± 1.5	98 ± 1.4
Crisp bread	6	85–94 ± 0.8	80–96 ± 0.5
Mysli	4	100 ± 0.1	96 ± 0.1
Rye flour	2	89 ± 0.6	99 ± 0.7
Diet	2	90 ± 0.1	96 ± 0.2
Potato	4	100–104 ± 0.8	90–97 ± 0.5
Meat	2	96 ± 1.3	97 ± 1.4
Milk	4	95 ± 0.6	93 ± 0.1

[a] Values are mean ± standard deviation.

The present method seems to be free of interferences, is applicable to various types of foods, and results in high recoveries. In addition, it is also suitable for routine work, because both thiamine and riboflavin can be extracted and cleaned up simultaneously and determined under the same chromatographic conditions, except for the wavelengths, which are changed between runs.

Acknowledgments

I thank Tuula Kurtelius and Marja-Terttu Wiisak for their skillful technical assistance.

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FOOD COMPOSITION AND ADDITIVES

Determination of Total Dietary Fiber in Foods and Products with Little or No Starch, Nonenzymatic–Gravimetric Method: Collaborative Study

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A collaborative study was conducted to validate a nonenzymatic–gravimetric method for the determination of total dietary fiber (TDF) of samples containing little or no starch such as most fruits, and vegetables and many purified polysaccharides. This simple procedure involves suspension of freeze-dried, ground samples in deionized water and incubation at 37°C for 90 min, followed by precipitation with 4 volumes of 95% ethanol. The weight of the dilute alcohol-insoluble residues after correcting for crude protein and ash corresponds to the TDF content of the sample. Six samples in blind duplicate (apples, apricots, cabbage, carrots, onions, and soy fiber) were sent with Celite to 10 laboratories. The reproducibility relative standard deviation (RSD_R) of the TDF values for 9 laboratories ranged from 2.92 to 6.25%. The repeatability standard deviation (RSD_r) for the 9 laboratories ranged from 1.50 to 2.70%. The method has been adopted first action by AOAC INTERNATIONAL.

The official AOAC enzymatic–gravimetric method, **985.29**, for the determination of total dietary fiber (TDF) has been used on a variety of foods and food products, including fruits, vegetables, and cereals (1). In an earlier study (2), we showed that it was not neccessary to include a protease digestion step for a number of selected foods. More recently, we found that for fruits and vegetables containing little (<2% dry weight) or no starch, the steps for gelatinization with Termamyl, and incubation with amyloglucosidase could also be eliminated. Starting with an alcohol-insoluble residue in a crucible, we followed the procedures as described in the AOAC method, and obtained very similar TDF values (3). The present collaborative study was conducted to determine the reproducibility and repeatability of the much simplified procedure.

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The recommendation was approved by the Committee on Food Nutrition and was adopted by the Official Methods Board of the Association. See "Changes in Official Methods of Analysis" (1994) *J. AOAC Int.* **77**, Jan/Feb issue and "Official Methods Board Actions" (1993) *The Referee*, **17**, July issue.

Collaborative Study

The 10 collaborating laboratories, including 1 Canadian, 1 Danish, and 8 American laboratories, are representatives from food manufacturers, government commercial testing laboratories, and universities. Six test samples in blind duplicates (4 g) and Celite (20 g) were sent to each laboratory. The 6 foods were (a) apples, (b) apricots, (c) cabbage, (d) carrots, (e) onions, and (f) FIBRIM 1450 (soy fiber), all of which had been used as test samples for other studies on dietary fiber analysis. Samples (b) through (e) were provided by Leon Prosky (4) of the U.S. Food and Drug Administration; sample (a) had been used in a study coordinated by Ruth Matthews of the U.S. Department of Agriculture, Human Nutrition Information Service, and sample (f) was obtained from Protein Technologies International, courtesy of Grace Lo. The freeze-dried samples were further dried 4 h in a vacuum oven at 60°C before bottling and shipping; samples were analyzed as received.

993.21 Total Dietary Fiber in Foods and Food Products with ≤ 2% Starch—Nonenzymatic-Gravimetric Method

First Action 1993

(Applicable to determination of ≥10% total dietary fiber in foods and food products with ≤2% starch, dry wt. basis.)

Method Performance:

See Table **993.21** for method performance data.

(*Caution*: See *Appendix: Laboratory Safety* for safe handling of organic solvents.)

A. Principle

Dried fruit, vegetable, or isolated fiber sources are suspended in H_2O and incubated 90 min at 37° to solubilize sugars and other water-soluble components. Water-soluble fiber components are then precipitated with ethanol. Residue is washed sequentially with 78% ethanol, 95% ethanol, and acetone and then dried at 105°. One duplicate is analyzed for crude protein, the other for ash. Total dietary fiber (TDF) is calculated as weight of residue less weight of protein and ash.

B. Apparatus

(**a**) *Analytical balance.*—Capable of weighing to 0.1 mg.

(**b**) *Air oven.*—Capable of maintaining 105 ± 0.5°.

(**c**) *Beakers.*—250 mL.

(**d**) *Desiccator.*—Containing mixture of colorless and indicating desiccant (Drierite is suitable).

(**e**) *Filtering flask.*—1 L capacity.

(**f**) *Fritted crucible.*—Porosity No. 2 (coarse ASTM 40–60 μm). Wet 0.5 g filter aid and evenly distribute by swirling with 78% ethanol; then apply vacuum to form even mat. Heat crucible containing filter aid in muffle furnace 1 h at 525°, let cool in desiccator, and weigh before use.

(**g**) *Incubator or waterbath.*—Capable of maintaining 37 ± 0.5°.

(**h**) *Muffle furnace.*—Capable of achieving 525°.

C. Reagents

(**a**) *Ethanol.—(1)* 95% (without any organic additive). *(2)* 78%. Dilute 207 mL H_2O with 95% ethanol to 1 L.

(**b**) *Acetone.*

(**c**) *Analytical filter aid.*—Acid-washed diatomaceous silica, ca 97.5% SiOH, ca 5% retained on 150 mesh screen [Celite®, Fisher Scientific, 711 Forbes Ave, Pittsburgh, PA; or C.A.F.A. (Celite Analytical Filter Aid), Manville Products Corp., PO Box 5108, Denver, CO, is suitable].

D. Determination

Accurately weigh to nearest 0.1 mg duplicate 500 mg freeze-dried, ground (≤30 mesh) samples or homogenized (by food processor) wet samples (containing ca 0.5 g dry matter) into separate 250 mL beakers. Add 25 mL (or volume necessary to bring wet sample to 25 mL) H_2O to each beaker; sonicate or gently stir suspensions until samples are thoroughly wet, i.e., no clumps remain. Scrape down any particles on inside wall of beaker with rubber policeman, and rinse walls with 1–2 mL H_2O. Cover beakers with Al foil and let stand 90 min without stirring in 37° incubator or water bath.

Add 100 mL 95% ethanol to each beaker and let stand 1 h at room temperature (25 ± 2°). Collect residue under vacuum in preweighed crucible containing filter aid. If and when filtration becomes very slow, use closed-end Luer needle, or any small pointed object, to gently scratch matted sample without disturbing filter aid. Positive pressure may also be used if available.

Wash residue 2× with 20 mL 78% ethanol, 2× with 10 mL 95% ethanol, and 1× with 10 mL acetone. Final rinsing with acetone should be done in fume hood, collecting acetone wash in separate filtering flask for proper disposal. Dry crucible containing residue ≥2 h at 105°. Cool crucibles ≥2 h in desiccator and weigh to nearest 0.1 mg.

Ash residue from one duplicate 5 h at 525°. Cool crucible ≥2 h in desiccator and weigh to nearest 0.1 mg.

Analyze residue from remaining duplicate for crude protein by Kjeldahl nitrogen determination, **960.52** or **992.15**, using %N × 6.25.

E. Calculations

Calculate TDF (%) as follows:

$$\text{TDF, \%} = 100 \times \frac{W_r - \frac{P+A}{100} \times W_r}{W_s}$$

where W_r = mg residue, P = % protein in residue, A = % ash in residue, and W_s = mg sample.

Ref.: JAOAC **77**, May/June issue (1994)

Results and Discussion

Ten laboratories had agreed to participate in this collaborative study, but one withdrew due to lack of time and personnel. The results of duplicate analyses of 6 test samples from 9 laboratories are shown in Table 1. All values were used for statistical evaluation (5). The average TDF (%) values, the repeatability relative standard deviation (RSD_r), and the reproducibility relative standard deviation (RSD_R) are given in Table **993.21**. The respective values are as follows for apples, 12.89, 1.72, 5.58; apricots, 26.56, 2.70, 5.12; cabbage, 26.56, 1.70, 2.92; carrots, 29.60, 1.50, 5.10; onions, 17.31, 2.58, 6.25; FIBRIM 1450, 76.66, 1.75, 3.60. Laboratory 4 was a Cochran outlier for carrots only because the within-laboratory variability was very small; therefore, data from all 9 laboratories were included in the final results. Comparisons of the average values from this study with those from our laboratory using the same procedures or an AOAC method (1) on the same test samples are presented in Table 2. This method is rapid and economical and has excellent precision both within- and between-laboratories.

Table 993.21 Method performance for total dietary fiber (TDF) in foods and food products with ≤2% starch by nonenzymatic-gravimetric method

Product	TDF, av. %	r	R	RSD_r, %	RSD_R, %
Apples	12.89	0.22	0.72	1.72	5.58
Apricots	26.56	0.72	1.36	2.70	5.12
Cabbage	26.56	0.45	0.78	1.70	2.92
Carrots	29.60	0.44	1.51	1.50	5.10
Onions	17.31	0.45	1.08	2.58	6.25
Soy polysaccharide	76.66	1.34	2.76	1.75	3.60

Table 1. Collaborative results of total dietary fiber (TDF) determination (%) by nonenzymatic-gravimetric method[a]

Sample	Lab. 1	Lab. 2	Lab. 3	Lab. 4	Lab. 5	Lab. 6	Lab. 7	Lab. 8	Lab. 9
Apples	12.44	12.87	12.21	12.82	13.18	12.31	13.11	14.29	12.08
	12.48	13.20	12.67	12.92	13.66	11.85	13.25	14.38	12.38
Apricots	25.05	27.16	27.64	29.01	26.99	24.45	26.85	27.21	25.31
	25.58	26.29	28.14	26.39	27.85	24.15	27.37	27.34	25.43
Cabbage	26.71	26.26	25.93	26.66	26.36	26.13	27.46	27.43	25.82
	25.99	26.98	26.25	27.74	26.95	25.25	27.52	27.38	25.33
Carrots	28.71	29.38	31.26	29.41	30.11	27.02	30.06	31.30	28.37
	28.43	29.58	31.66	31.03	29.97	26.49	30.40	31.10	28.60
Onions	16.62	17.03	18.36	17.98	17.84	16.24	17.64	17.20	16.04
	16.55	16.93	18.83	19.59	18.29	15.61	17.36	17.49	16.09
FIBRIM	74.07	76.55	79.06	77.09	75.59	71.94	80.49	80.03	73.96
	75.01	78.36	76.49	76.48	78.84	72.97	81.61	77.14	74.24

[a] Blind duplicates.

Recommendation

It is recommended that this nonenzymatic–gravimetric method for the determination of total dietary fiber be adopted first action.

Acknowledgments

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Table 2. Total dietary fiber (TDF; g/100 g dry wt) in test samples: method comparison[a]

Sample	This study	Nonenzymatic–gravimetric	Phosphate buffer
Apples	12.89	13.00 ± 0.63	11.50 ± 0.22
Apricots	26.56	25.70 ± 0.71	24.14 ± 0.07
Cabbage	26.56	26.12 ± 0.01	24.68 ± 0.63
Carrots	29.60	28.70 ± 0.03	24.39 ± 1.12
Onions	17.31	16.72 ± 0.06	16.30 ± 0.26
FIBRIM	76.66	79.53 ± 0.51	77.17 ± 0.64

[a] Values (mean of duplicate determinations ± SD) from Nutrient Composition Laboratory using nonenzymatic–gravimetric method (**993.21**) (6) and phosphate buffer method (993.19) (6).

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FOOD COMPOSITION AND ADDITIVES

Determination of Soluble Dietary Fiber in Foods and Food Products: Collaborative Study

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A collaborative study was conducted to determine the soluble dietary fiber (SDF) content of foods and food products by using a combination of enzymatic and gravimetric procedures. The method was basically the same as that employed for determining total dietary fiber (TDF), 985.29, and the method for insoluble dietary fiber (IDF), 991.42. Ten laboratories were each sent 13 test samples (6 blind duplicates and 1 standard [green beans] containing 29–33% TDF, 19–23% IDF, and 8–13% SDF) and were instructed to assay for IDF, SDF, and TDF independently. Included in the package were the 3 enzymes, namely alpha-amylase, amyloglucosidase, and protease, and the filter aid Celite, which was thought to be the major cause of high reproducibility relative standard deviation (RSD_R) values for SDF obtained in a previous collaborative study. The foods to be analyzed were apricots, carrots, chickpeas, onions, raisins, and the sugar beet fiber Fibrex™. IDF, TDF, and SDF were calculated as the weight of residue minus the weight of protein and ash on a dry weight basis. RSD_R values of the IDF results averaged 8.02%, with only 1 food having an RSD_R >10%. The RSD_R values for the TDF results averaged 4.97%, and all foods had an RSD_R <7%. Although the RSD_R values for SDF averaged 14.17%, 4 of the 6 foods had an RSD_R <10%, and 1 of the 2 remaining foods that had a high RSD_R had an SDF content of only 1.2%. In all cases, the RSD_R values of the SDF content of the foods were less than the values for the same foods analyzed in a previous collaborative trial. The enzymatic-gravimetric method for the determination of SDF was adopted first action by AOAC INTERNATIONAL.

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The enzymatic-gravimetric determination of total dietary fiber (TDF) in foods, **985.29**, was adopted final action by AOAC (1); the enzymatic-gravimetric method for insoluble dietary fiber (IDF), **991.42**, was adopted first action by AOAC (2). In a preliminary study it was found that the basic method for determination of TDF could be modified to measure IDF by filtering out the IDF before precipitating the soluble dietary fiber (SDF) with ethanol; this method was described earlier in a similar method using physiological enzymes (3) and in 2 previous collaborative studies (4, 5).

In the previous study (5), 22 foods were analyzed for SDF. The products had an average SDF value that ranged from 1.35% for chickpeas to 33.42% for prunes. The reproducibility relative standard deviation (RSD_R) values for half the foods analyzed were higher than 20%. Because it was suspected that the large variation was due in part to the variations in the Celite used, extra precautions were taken in the present study to obtain a specific Celite preparation of uniform quality. Also, 5 of the 6 samples chosen for collaborative study were taken from foods that gave high RSD_R values in the previous study (5). The present study was designed according to the rules of Youden and Steiner (6).

Collaborative Study

The collaborators from the 10 laboratories participating in this study, representing 8 countries, were analysts in food companies, universities, and commercial and government laboratories. Each laboratory was sent 13 test samples (6 blind duplicates and 1 standard) for determination of TDF, IDF, and SDF. The standard, green beans, contained 29–33% TDF, 19–23% IDF, and 8–13% SDF. These values were derived from a previous collaborative study. The collaborators were also sent the 3 enzymes used in the method, namely, alpha-amylase, amyloglucosidase, and protease, as well as the filtering aid Celite. The collaborators were further instructed to weigh test portions to the nearest 0.1 mg and to calculate % TDF, % IDF, and % SDF to 2 decimal places according to the formulas provided.

The following 6 foods were to be analyzed for TDF, IDF, and SDF: (*a*) apricots, (*b*) carrots, (*c*) chickpeas, (*d*) onions, (*e*) raisins, and (*f*) sugar beet fiber. Items (*a*) and (*e*) were supplied by Vacu-Dry, Santa Rosa, CA; item (*b*) was supplied by California Vegetable Concentrate, Modesto, CA; item (*c*) was purchased as the dried material in a local supermarket; item (*d*) was supplied by Basic American Foods, San Francisco, CA; and item (*f*), sugar beet fiber (Fibrex), was graciously donated to us by Delta Fibre Foods, Minneapolis, MN.

To prepare test samples, all products were homogenized in water in a kitchen food processor, lyophilized, ground in a continuous-grinding Microjet 10 Centrifugal Mill (Quartz Technology, Inc., Westbury, NY) to a uniform size, and treated as described previously (5). None of the test samples contained more than 10% fat; therefore, fat extraction was not recommended.

The collaborators performed a moisture analysis before the determination of TDF, IDF, and SDF so that the results could be reported on a dry matter basis (Table 1).

993.19 Soluble Dietary Fiber in Food and Food Products—Enzymatic-Gravimetric Method (Phosphate Buffer)

First Action

[Applicable to determination of soluble dietary fiber (SDF) in vegetables, fruit, and cereal grains and to determination of total dietary fiber (TDF) in conjunction with **991.42**, Insoluble Dietary Fiber (IDF) in Food and Food Products.]

Method Performance

See Tables **993.19A**, **993.19B**, and **993.19C** for method performance data.

(*Caution*: See *Appendix: Laboratory Safety* for safe handling of organic solvents.)

A. Principle

Duplicate test portions of dried foods, fat-extracted if >10% fat, are gelatinized with heat-stable *alpha*-amylase and then enzymatically digested with protease and amyloglucosidase to remove protein and starch. IDF is removed by filtering and washing residue with water. SDF in filtrate is precipitated by adding 95% ethanol to filtrate. Precipitate is filtered and washed with 78% ethanol, 95% ethanol, and acetone; dried; and weighed. One duplicate is analyzed for protein, and the other is incinerated at 525° to determine ash. SDF is weight of residue minus weight of protein and ash.

B. Apparatus

See **991.42B**.

C. Reagents

See **991.42C** with following change: Reagent (**j**), Celite, medium grade (acid-washed) (Sigma Chemical Co., St. Louis, MO, is suitable source).

D. Enzyme Purity

See **991.42D**.

E. Preparation of Samples

Analyze dry foods without pretreatment whenever possible. Dry-mill to 0.3–0.5 mm mesh. Homogenize and freeze-dry wet foods before milling. If high fat content (>10%) prevents proper milling, defat with three 25 mL portions of petroleum ether/g food before milling. Determine residual moisture in milled foods by drying overnight in 70° vacuum oven or 5 h in 105° air oven. Record weight loss due to fat and/or water, and make appropriate correction to final % TDF, IDF and SDF. (*Note*: For foods high in sugars that cannot be dried by lyophilization, extract test sample 3 times each with 10 volumes 85% methanol to remove sugars before milling or lyophilization, which may interfere in determination.)

F. SDF Determination

Proceed as in **991.42F**, from beginning up through "Wash residue...2 times with 10 mL acetone." in paragraph 5.

Adjust weight of combined filtrate and water washings to 100 g with H_2O. Add 4 volumes (400 mL) 95% ethanol, preheated to 60°. Let precipitate form at room temperature 60 min.

Tare crucible containing Celite to nearest 0.1 mg; then wet and redistribute Celite bed in crucible, using stream of 78% EtOH from wash bottle. Apply suction to crucible to draw Celite onto fritted glass as even mat.

Filter precipitate mixture and wash residue successively with three 20 mL portions of 78% EtOH, two 10 mL portions of 95% EtOH, and two 10 mL portions of acetone.

Proceed as in **991.42F**, starting with "Break surface film..." in paragraph 5 through end of **991.42F**.

G. Calculation

Determination of blank:

$$B = \text{blank, mg} = \text{wt residue} - P_B - A_B$$

where wt residue = av. of residue wts (mg) for duplicate blank determinations; and P_B and A_B = wts (mg) of protein and ash, respectively, determined in first and second blank residues.

Table 1. Collaborative study results (blind duplicates) of determination of total dietary fiber (TDF), insoluble dietary fiber (IDF), and soluble dietary fiber (SDF) by enzymatic-gravimetric method

Lab.	TDF, %		IDF, %		SDF, %	
Apricots						
1	24.68	23.36	—[a]	—	—	—
2	25.61	24.98	12.84	13.82	11.98	12.25
3	26.00	24.26	15.60	13.68	11.23	—
4	25.67	25.88	—	—	—	—
5	25.06	24.82	13.49	14.11	11.74	11.24
6	24.23	24.85	13.31	13.28	11.53	11.57
7	25.19	25.24	13.45	15.20	10.98	9.90
8	24.76	24.56	12.72	12.68	12.40	12.24
9	24.48	22.39	15.45	15.93	10.47	10.56
10	22.79	23.82	13.17	13.56	9.48	10.47
Carrots						
1	22.99	22.43	14.31	13.56	9.01	10.44
2	23.88	23.50	10.31	12.28	12.60	11.28
3	23.24	21.39[b]	14.24	13.26	10.75	11.32
5	24.03	23.89	13.95	14.15	11.29	11.01
6	23.15	23.37	11.52	11.59	12.20	12.20
7	23.46	23.82	12.31	12.60	11.93	11.64
8	24.00	24.12	11.32	11.36	13.56	13.28
9	23.38	23.56	14.13	13.36	10.61	10.76
10	21.92	22.33	11.60	12.03	12.21	11.39
Chickpeas						
1	17.38	14.65	17.45	13.34	0.95	—
2	14.35	13.31	15.35	15.07	1.41	0.65
3	13.78	14.76	16.60	15.75	0.86	1.00
4	14.01	14.57	13.07	14.48	0.81	0.99
5	15.33	14.34	18.93	15.93	1.16	—
6	13.42	14.42	11.80	11.66	1.83	1.70
7	13.40	14.16	15.34	16.49	1.23	1.05
8	13.26	12.88	11.96	11.70	1.64	1.54
9	14.46	14.12	12.32	13.95	1.49	1.40
10	15.65	14.30	16.54	16.59	1.01	1.46
Onions						
1	17.23	15.84	12.42	11.63	4.14	3.63
2	16.38	16.68	12.48	13.50	2.91	3.83
3	16.07	16.16	12.31	11.86	5.21	2.00
5	16.23	17.68	11.73	11.95	5.22	5.35
6	16.15	16.54	13.54	13.14	3.95	3.74
7	16.75	16.22	12.40	13.43	4.25	3.77
8	16.58	16.26	—	—	—	—
9	12.79	16.14[c]	11.22	11.85	5.45	5.26
10	15.56	15.03[c]	12.62	14.66	4.24	3.07
Raisins						
1	31.15	—	27.13	—	7.54	—
2	29.97	28.70	23.22	22.38	—	—
3	31.22	30.73	22.65	22.23	7.46	7.71
4	33.00	34.69	22.26	24.08	7.86	7.38
5	30.22	30.76	21.95	21.40	8.75	8.57
6	29.17	29.87	21.83	22.97	8.56	7.55
7	31.64	31.41	22.05	23.96	9.24	7.23
8	30.20	30.22	22.40	22.22	7.78	7.76
9	29.42	29.60	22.37	21.38	7.64	8.57
10	27.54	25.01	24.56	22.53	4.97	4.02[d]
Sugar beet fiber						
1	67.60	64.30	45.26	46.83	20.25	18.12
2	65.98	65.49	45.66	45.60	22.26	21.22
3	66.39	66.12	44.46	44.28	21.71	21.96
4	68.80	68.27[c]	46.80	47.13	20.44	19.61
5	66.74	66.74	46.78	46.44	21.80	20.39
6	65.80	65.97	44.71	44.92	21.91	22.19
7	65.63	68.03	45.09	46.38	22.49	20.66
8	65.94	65.86	44.96	45.00	19.24	19.94
9	64.50	67.38	46.33	45.53	20.96	20.52
10	63.49	62.49[c]	43.57	45.58	18.93	18.31

[a] No results reported.
[b] Cochran outlier, used in statistical calculations.
[c] Paired Grubbs outlier, used in statistical calculations.
[d] Grubbs outlier, not used in statistical calculations.

Calculate SDF as follows:

$$SDF, \% = \frac{\text{wt residue} - P - A - B}{\text{wt sample}} \times 100$$

where wt residue = av. of wts (mg) for duplicate sample determinations; P and A = wts (mg) of protein and ash, respectively, in first and second sample residues; and wt sample = av. of 2 sample wts (mg) taken.

Calculate TDF as follows:

$$\text{TDF, \%} = \text{SDF} + \text{IDF(from } \mathbf{991.42})$$

Ref.: JAOAC **77**, May/June issue (1994)

Results and Discussion

The determination of TDF by an enzymatic-gravimetric method was adopted final action by AOAC in 1986 and modified in 1988 (4). The modified method was also given final approval by the American Association of Cereal Chemists in 1992 (7) and used in a collaborative study for IDF and SDF in which the IDF method was adopted first action by AOAC (5). In that study of 22 foods, the determination of SDF showed promise; 11 of the foods had RSD_R values <20%. However, the remaining foods had high RSD_R values that could not necessarily be associated with low SDF results. Preliminary results for the SDF determination indicated that a major reason for the differences between laboratory results was the long and vari-

Table 993.19A. Method performance for soluble dietary fiber (SDF) in foods by enzymatic-gravimetric method (phosphate buffer)

Food	No. of labs	SDF av., %	s_r	s_R	RSD_r, %	RSD_R, %
Apricots	8	11.20	0.42	0.91	3.78	8.11
Carrots	9	11.53	0.53	1.11	4.59	9.61
Chickpeas	10	1.21	0.23	0.34	19.52	28.28
Onions	8	4.13	0.91	1.00	21.93	24.12
Raisins	8	7.95	0.67	0.67	8.41	8.41
Sugar beet fiber	10	20.65	0.80	1.35	3.88	6.52

Table 993.19C. Method performance for insoluble dietary fiber (IDF) in foods by enzymatic-gravimetric method (phosphate buffer)

Food	No. of labs	IDF, av. %	s_r	s_R	RSD_r, %	RSD_R, %
Apricots	8	13.89	0.73	1.08	5.24	7.80
Carrots	9	12.66	0.59	1.27	4.68	10.03
Chickpeas	10	14.72	1.28	2.19	8.70	14.86
Onions	8	12.55	0.69	0.91	5.53	7.29
Raisins	10	23.03	0.90	1.37	3.91	5.95
Sugar beet fiber	10	45.57	0.67	0.98	1.48	2.17

able filtration times, both when the IDF fraction was removed from the SDF fraction by filtration and when the precipitated SDF fraction was filtered in the second filtration step. Several laboratories suggested that these problems could be overcome by using 0.5–0.25 g test portions for analysis of materials containing high levels of viscous fiber, which hinder filtration. The high variability in the determination of SDF was also due in part to the lack of uniformity of the Celite used by the collaborators. We therefore sought to minimize this problem by sending the collaborating laboratories a specific Celite preparation of uniform quality (same lot number) and instructions for preparation before use.

All 10 laboratories submitted results. In a few cases, no results were reported because the investigator did not have sufficient test sample (apricots and onions, IDF and SDF), and in other cases some of the analytes were lost during analysis (chickpeas, SDF; raisins, 1 of a pair of duplicates of TDF, IDF, and SDF).

The results of the individual determinations for TDF are shown in Table 1. For the TDF determination, only 1 laboratory's value was a Cochran standard statistical outlier for 1 food (carrots), and 2 laboratories' values were paired Grubbs outliers for onions and sugar beet fiber. All values were used in the statistical calculation, however. All other values were used as reported by the analysts. The measures of precision for TDF are shown in Table **993.19B**. The average TDF values ranged from 14.33% for chickpeas to 66.07% for sugar beet fiber. The repeatability relative standard deviation (RSD_r) for the determination of TDF of the 6 foods analyzed ranged from 1.74% for sugar beet fiber to 5.93% for chickpeas. The reproducibility relative standard deviation (RSD_R) ranged from 2.41% for sugar beet fiber to 7.01% for chickpeas. The RSD_R values for the 6 foods averaged 4.98%, which is considered excellent for this determination and a considerable improvement over our results in the 1988 collaborative study (4).

Table 993.19B. Method performance for total dietary fiber (TDF) in foods by enzymatic-gravimetric method (phosphate buffer)

Food	No. of labs	TDF av., %	s_r	s_R	RSD_r, %	RSD_R, %
Apricots	10	24.63	0.74	0.97	3.03	3.95
Carrots	9	23.25	0.49	0.79	2.10	3.38
Chickpeas	10	14.33	0.85	1.00	5.93	7.01
Onions	9	16.13	0.95	1.02	5.88	6.33
Raisins	10	30.28	0.81	2.05	2.69	6.78
Sugar beet fiber	10	66.07	1.15	1.59	1.74	2.41

The results of the individual determinations of IDF are shown in Table 1. Because there were no statistical outliers, all values were used as reported. The measures of precision for the determination of IDF are shown in Table **993.19C**. The average IDF values for the products analyzed ranged from 12.55% for onions to 45.57% for sugar beet fiber. RSD_r values ranged from 1.48% for sugar beet fiber to 8.70% for chickpeas. RSD_R values ranged from 2.17% for sugar beet fiber to 14.86% for chickpeas, with an average RSD_R of 8.02% for the 6 foods. This compares very favorably with the average RSD_R for IDF of 11.09% in the previous collaborative study in which the IDF method was adopted official first action.

The results of the individual determinations of SDF are also shown in Table 1. Only 1 value was a Grubbs outlier and was not used in the statistical calculations. The measures of precision for the determination of SDF are shown in Table **993.19A**. The products analyzed had average SDF values that ranged from 1.21% for chickpeas to 20.65% for sugar beet fiber. RSD_r values ranged from 3.78% for apricots to 21.93% for onions. RSD_R values ranged from 6.52% for sugar beet fiber to 28.28% for chickpeas. There was, however, a substantial reduction in the RSD_R values, in all cases, when these results were compared with those from the previous collaborative study (4). The RSD_R value for apricots was decreased from 16.36 to 8.11%, for carrots from 15.76 to 9.61%, for chickpeas from 44.38 to 28.28%, for onions from 37.86 to 24.12%, and for raisins from 41.21 to 8.41%.

Table 2 shows the method performance for the ratio of TDF determined by the sum of IDF and SDF to the independent determination of TDF. The ratio varied from 1.00 for sugar beet fiber to 1.10 for chickpeas, with an average ratio of 1.03. The RSD_R values ranged from 2.27% for sugar beet fiber to 11.46% for onions, with an average RSD_R of 6.85%. This means that,

Table 2. Method performance for ratio of total dietary fiber (TDF) determined by sum of insoluble dietary fiber (IDF) and soluble dietary fiber (SDF) to independent determination of TDF

Food	No. of labs	Ratio, (IDF + SDF) /TDF[a]	RSD_r, %	RSD_R, %
Apricots	8	1.02	4.05	5.26
Carrots	9	1.03	4.25	4.93
Chickpeas	9	1.10	5.77	11.15
Onions	9	1.02	8.49	11.46
Raisins	8	1.01	1.44	6.00
Sugar beet fiber	9	1.00	2.13	2.27

[a] Independent determination.

to determine TDF, the final action method for TDF can be used or the values determined for SDF and IDF can be summed.

Recommendation

We recommend that the enzymatic-gravimetric method for the determination of soluble dietary fiber be adopted first action. This method can be used to determine total dietary fiber in conjunction with the official first action method for insoluble dietary fiber. The final action method for total dietary fiber may be used when only the total is required.

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FOOD COMPOSITION AND ADDITIVES

Determination of Honey Authenticity by Anion-Exchange Liquid Chromatography

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Methodology using anion-exchange liquid chromatography with pulsed amperometric detection was developed to determine the addition of invert syrups (beet or cane) and high-fructose corn syrup to honey. The invert syrups used were either chemically (commercially) or enzymatically prepared. Fingerprint oligosaccharides were shown to be present in these sweeteners, which were either not detectable or present at low concentrations in pure honey. Forty-four pure honey samples produced in continental North America, Hawaii, China, and Australia were used in this study.

The determination of food authenticity has long been a problem in the food industry (1). This problem has become more complex as the types of adulterants added to food have become more sophisticated. The advent of modern analytical instruments has greatly enhanced the ability of regulatory agencies to detect many types of adulteration. Despite these technological advances, there is still a problem with the adulteration of high-carbohydrate foods, such as honey, with inexpensive syrups. The most common syrups used to adulterate honey are invert syrups (IS), produced from cane or beet sucrose, and high-fructose corn syrup (HFCS). These syrups are inexpensive, and their carbohydrate profiles can be manipulated easily to resemble the carbohydrate profile of honey.

Several methods have been developed to detect HFCS addition to honey: carbohydrate analysis by gas-liquid (GLC), thin-layer (TLC), and liquid chromatography (LC); sodium/potassium ratio by atomic absorption analysis; immunochemical analysis; characterization of honey proteins by gel electrophoresis; analysis of pollen and proline; differential scanning calorimetry; and turbidimetry and colorimetry (for reviews, see references 2 and 3).

An important breakthrough in the detection of HFCS in honey resulted from the discovery by Smith and Epstein (4) and Bender (5) that the $^{13}C/^{12}C$ ratio in organic compounds varied among certain types of plants. Their studies revealed that plants such as corn and sugar cane, which use the Hatch–Slack (C_4) photosynthetic cycle, had lower amounts of ^{13}C than plants using the Calvin (C_3) photosynthetic cycle. Nissenbaum et al. (6) applied this information to detect the addition of corn products to various foods. The $^{13}C/^{12}C$ ratio has been used to detect the addition of HFCS to honey (7), and analysis times have been shortened because of technological advances (8). Analysis of $^{13}C/^{12}C$ ratio works well but is costly, time consuming, and relatively insensitive because some honeys may contain as much as 25% HFCS and still fall within the normal range (9). However, a modification of this procedure using honey protein as an internal standard, introduced by White and Winters (9), seems to offer some improvement in the detection levels of HFCS in honey (reported to be approximately 7%). In addition to the problems already mentioned, analysis of $^{13}C/^{12}C$ ratio would not detect a high-fructose syrup made from starch derived from C_3 plants, such as potatoes.

Attempts have been made to detect HFCS in honey by carbohydrate analysis using LC (10), TLC, and GLC (11). Variations in carbohydrate profiles in pure honeys reduced the effectiveness of these tests. Lipp et al. (12) introduced an LC method to detect HFCS in honey by analysis of glucose polymers. The authors claimed detection of low levels of HFCS in honey (1%), although they only analyzed 1 honeydew and 1 nectar honey. In addition, recent changes in the production of HFCS have reduced the levels of glucose polymers. This fact, coupled with the need to analyze many more honey samples, clearly limits the usefulness of this method. The presence of 5-(hydroxymethyl)-2-furaldehyde (HMF) had been used as a test for the addition of beet sugar hydrolysates to honey. This test was reviewed (13), and a content of 200 ppm was suggested as that requiring further examination by other methods. HMF is formed during acid hydrolysis of sucrose from sugar beets and sugar cane. The drawback with this test is that HMF is also naturally present in honey, especially if it has been subjected to heat or abusive storage (14). Results from our laboratory indicate that the levels of HMF in commercial invert syrups may be low (<76 ppm; unpublished results). In addition, variations in the HMF content of honeys and invert syrups make this method useful as a screening tool only. The use of galactose oxidase was developed by White et al. (15) to detect beet invert syrups (BIS) in honey. This procedure is useful only as a screening tool because of wide variation in bound galactose in BIS and honey. Before our research, there was no de-

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finitive method that could be used to determine the presence of any level of BIS in honey (13, 15).

It has been well documented that the invertase activity in honey originates from bees (16). In addition, research has revealed that carbohydrate hydrolytic enzymes in honey exhibit both α-glucosidase (17) and β-glucosidase (18) activity. During the ripening of honey, the enzyme responsible for most hydrolytic activity is invertase, which not only hydrolyzes sucrose but also catalyzes the formation of several oligosaccharides via transglycosylation reactions (17). Several researchers have observed this enzyme-catalyzed formation of oligosaccharides via transglucosylation–transfructosylation reactions in a number of natural foods (17–20). The oligosaccharides formed during the natural hydrolysis of sucrose in honey during the ripening process would contain α-and β-glucosyl units, because the only hydrolase enzymes found in honey exhibit glucosidase activity (17, 18).

Invert syrups can be produced by the acid- and/or enzyme-catalyzed hydrolysis of beet or cane sucrose. The glucose/fructose/sucrose ratio can be regulated by controlling the amount of hydrolysis that takes place. Because the amount of sucrose in honey is less than 8% (21–25), nearly complete hydrolysis of beet or cane sucrose must be obtained to achieve the proper glucose/fructose/sucrose ratio.

The main commercial sources of invertase, which could be used to hydrolyze sucrose for the production of invert syrups, are the yeasts, *Saccharomyces cerevisiae* and *Saccharomyces uvarum* (26). Enzymes from both yeasts exhibit β-fructosidase activity (27), while the hydrolase enzymes present in honey are α- and β-glucosidases (17, 18). Although both invertases hydrolyze sucrose, each type will catalyze the synthesis of different transglycosylation oligosaccharides.

Invert syrups can be produced also by the controlled acid hydrolysis of sucrose. The production of oligosaccharides by acid-catalyzed transglycosylation reactions during sucrose hydrolysis is called reversion (28). Unlike the enzyme transglycosylation reaction where there is no change in configuration, reversion reactions can result in the formation of both α and β anomers (28).

The majority of the oligosaccharides in HFCS result from the incomplete enzymatic hydrolysis of starch. These oligosaccharides are composed only of glucose molecules linked α-1→4 and/or α-1→6 (29). Oligosaccharides may be synthesized also by α- and β-amylase, pullulanase, and glucoamylase during starch hydrolysis via transglucosylation reactions (30).

This paper presents methodology for the detection of invert syrups and high-fructose corn syrup in honey by the analysis of trace oligosaccharides via anion-exchange liquid chromatography with pulsed amperometric detection.

Experimental

Thirty-eight pure honeys were gifts from the U.S. National Honey Board. The honeys originated in Canada, Mexico, continental United States, Hawaii, Australia, and China. Unifloral Alfalfa, Alsike, Canola, Red Clover, Sweet Clover, and Trefoil honey samples were obtained by our laboratory. The origin and collection of these pure honeys is described elsewhere (24). All samples were stored at–20°C until required for analysis.

Cane invert syrup (IS-C1) was a gift from Tate and Lyle, U.K. Two beet invert syrups (IS-C2 and IS-C3) were gifts from Canadian Blending and Processing, Inc. (Windsor, ON); and 2 beet invert syrups (IS-C4 and IS-C5) were gifts from Redpath Sugars (Toronto, ON) and Lantic Sugar Ltd. (Toronto, ON), respectively. The high-fructose corn syrups (55% fructose) were gifts from Canada Starch Company Inc., Casco, London, ON (HFCS1); ADM Corn Processing, Decatur, IL (HFCS2); CPC International Inc., Argo, IL (HFCS3); and American Maize Products Co., Decatur, AL (HFCS4).

A crude commercial β-fructosidase (Sigma Chemical Co.) was used to enzymatically produce an invert syrup (IS-E). The invertase (28 000 units) was dissolved in a minimum volume of LC-grade H_2O. The resulting solution was added to 100.0 g of a 75.7 °Brix beet sucrose solution (Alberta Sugar Company). The solution was maintained at pH 5.0 and 55°C with constant stirring. Ten gram aliquots were removed every 10 min, heated to 80°C for 5 min to terminate the reaction, and analyzed by LC to monitor hydrolysis. The sample containing <5% sucrose (80 min) was used as an adulterant in this study.

The water content of each honey and sweetener sample was determined by using a Karl Fischer-Automat titrator (Metrohm, Model 633). Fresh methanol was blanked with pyridine-free Karl Fischer reagent (BDH, Comp-5 Aquastar) before addition of the sample. A 20 s delay was set on the titrator to ensure a stable end point. Ten microliters of water was used as the standard, and ca 50 mg of sample was used for each titration. Triplicate moisture analyses of the honeys and sweeteners were performed.

Sample preparation for glucose, fructose, and sucrose quantitation was achieved by simple dilution of each honey and sweetener with deionized water. Approximately 0.200 g of each sample was weighed accurately into a 1 L volumetric flask and passed through a 0.22 μm sterile Millex GS filter (Millipore) to remove particulate matter before LC analysis. Standard curves of glucose, fructose, and sucrose were constructed to determine their response factors during LC analysis. The concentration of these standards ranged from 0.013 to 0.154 mg/mL. Concentrations of glucose, fructose, and sucrose in the honey and sweetener samples were calculated from standard curves. Statistical analysis of the standard curves showed a correlation coefficient of 0.983 or better.

Sample Preparation

Monosaccharides were removed from the samples (pure honeys, adulterants, and adulterated honeys) by modifying a procedure from Whistler and Durso (31). One gram of each sample was diluted in 19.0 g of deionized water and stirred with 4.0 g of activated charcoal, 50–200 mesh (Fisher Scientific Co.), for 17 h at 4°C. After mixing, the samples were placed on a 3.0 × 40.0 cm column containing 4.0 g of activated charcoal–Celite (50 + 50, w/w; Fisher Scientific Co.). More than 95% of the monosaccharides were removed from the column (estimated by LC) by washing with 1 L of 0.1% (v/v) ethanol at room temperature and at a flow rate of 10.0 mL/min

maintained by vacuum. The oligosaccharides were eluted from the column with 500 mL of a 60°C solution of 50% (v/v) ethanol at the same flow rate. The filtrate was dried at 35°C in a rotary evaporator (Buchi) and dissolved in 10 mL of water. Ninhydrin and Bradford tests were carried out to ensure that contamination of the samples with amino acids and proteins was below 0.5 mg/mL and 20 μg/mL, respectively. The samples were stored at −20°C until required for analysis.

To ensure that no loss of oligosaccharides had occurred during removal of glucose and fructose, a solution containing sucrose, maltose, and maltotriose was placed on the charcoal–Celite column. The column was washed with 0.1% ethanol at room temperature and 50% ethanol at 60°C as described earlier, and both eluates were saved. Analysis of the eluates by LC revealed that >99% of the sucrose, maltose and maltotriose was recovered during the charcoal–Celite treatment. In addition, the 0.1% ethanol wash was analyzed by LC to ensure that there was no loss of oligosaccharides.

To ensure that the charcoal–Celite procedure did not cause variations in oligosaccharide concentrations, multiple preparations of a single, pure honey sample were carried out by the method described earlier. Analysis of the carbohydrates by LC revealed that there were few or no changes in concentration of the oligosaccharides in the 50% ethanol wash from one preparation to the next.

There were 2 methods used to adulterate the honey samples. Initially, the honey and sweetener samples were standardized to the same °Brix. The honey sample was then mixed with the sweetener at a certain level on a w/w basis. This adulterated honey sample was treated with charcoal–Celite as described earlier. It was later found that the honey and sweetener samples could be standardized to the same °Brix and individually prepared by using the charcoal–Celite treatment. After this treatment, the honeys were adulterated by addition of the sweeteners to a certain level on a weight basis. LC analysis of several adulterated honey samples prepared by both methods revealed that there were few or no variations in oligosaccharide concentrations in the samples.

Oligosaccharide Analysis by LC

Separation of the oligosaccharides by LC was carried out on a Waters 625 LC system with 2 Dionex 10 μm Carbo Pac PA1 pellicular anion-exchange columns (4 × 250 mm) connected in series with a mobile-phase flow rate of 0.70 mL/min. The carbohydrates were detected by a PAD (pulsed amperometric detector; Waters, Model 464) with a dual gold electrode at a sensitivity of 50 μA. The working electrode was maintained at the following potentials and durations during operation: $E_1 = 0.05$ V ($t_1 = 0.299$ s); $E_2 = 0.60$ V ($t_2 = 0.299$ s); $E_3 = 0.80$ V ($t_3 = 0.499$ s). A postcolumn delivery system (Scientific Systems Inc., Model 350) of 300 mM sodium hydroxide (NaOH) at a flow rate of 0.80 mL/min was used to minimize baseline drift. The carbohydrates eluting from the columns were plotted by a Maxima 820 chromatography work station (Millipore). All injections were done in duplicate to ensure reproducible peak retention times and areas.

Table 1. Gradient elution used in the detection of IS-C and IS-E in honey (method 1)[a]

Time, min	A, %	B, %	C, %
0	100	0	0
4.0	100	0	0
20.0	97	3	0
75.0	0	100	0
75.1	100	0	0
85.1	100	0	0
85.2	0	0	100
95.0	0	0	100
95.1	100	0	0
135.0	100	0	0

[a] A = 100mM NaOH; B = 100mM NaOH and 100mM sodium acetate (NaOAc); C = 300mM NaOH.

The LC gradient elution program used for the detection of chemically produced invert syrup (IS-C) and enzymatically produced invert syrup (IS-E) in honey is listed in Table 1 (method 1). The sample volume for this method was 20 μL.

The LC method used for the detection of HFCS in honey employed one Dionex 10-μm Carbo Pac PA1 pellicular anion-exchange column (4 × 250 mm) with a mobile-phase flow rate of 1.00 mL/min. Table 2 lists the LC gradient elution program used for the detection of HFCS in honey (method 2). The sample volume for this method was 600 μL.

Results and Discussion

The geographical and botanical origins of the 44 pure honey samples; their glucose, fructose, and sucrose contents; fructose/glucose ratio; and water content are listed in Table 3. The minor oligosaccharides of the pure honeys were analyzed by LC (method 1) after removal of >95% of the monosaccharides by charcoal–Celite chromatography. Figures 1 and 2 are LC chromatograms of 2 pure honeys (samples 30 and 35) that illustrate the complex mixture of carbohydrates in this food. LC analysis of each of the pure honey samples in this study revealed that these honeys were representative. Although the oligosaccharide profile of each of the pure honey samples ana-

Table 2. Gradient elution used in detection of HFCS in honey (method 2)[a]

Time, min	A, %	B, %	C, %
0	100	0	0
5.0	100	0	0
37.0	0	100	0
38.0	0	0	100
67.0	0	0	100
68.0	100	0	0
113.0	100	0	0

[a] A = 100mM NaOH; B = 100mM NaOH and 250mM NaOAc; C = 300mM NaOH.

Table 3. Geographical and botanical origin[a] of 44 pure honeys and their glucose (G), fructose (F), and sucrose (S) contents, F/G ratios, water contents, and areas of marker peaks I, II, and III

Sample No.	Origin of honey sample	Botanical origin	G, %	F, %	S, %	F/G ratio	Water, %	Peak areas ($\times 10^5$)		
								Peak I	Peak II	Peak III
1	Alberta	Clover	36.6	40.7	0.3	1.11	17.0	3.60	2.16	2.65
2	Alberta	Alfalfa	33.9	37.0	1.2	1.09	19.6	ND[b]	2.40	0.66
3	Alberta	Alsike	35.6	38.5	0.9	1.08	17.1	ND	ND	0.50
4	Alberta	Canola	40.0	36.2	0.1	0.91	18.9	ND	ND	0.48
5	Alberta	Clover	33.2	37.1	0.6	1.12	21.9	ND	0.95	ND
6	Alberta	Clover	36.7	36.7	0.7	1.00	18.7	ND	ND	0.95
7	Alberta	Trefoil	34.8	38.6	0.9	1.11	19.2	ND	0.42	0.84
8	China	Acacia	28.0	45.2	1.2	1.61	18.2	1.10	4.34	2.03
9	Eastern Australia	Orange	30.7	41.2	2.7	1.34	16.0	3.47	3.82	4.14
10	Georgia	Tupelo	25.3	46.1	3.9	1.82	16.3	ND	3.49	2.78
11	Hawaii	Kiawe	31.7	39.1	2.1	1.23	16.6	2.59	2.81	3.37
12	Hawaii	Lilikow Macadamia Lychee Clover	34.3	41.6	1.6	1.21	18.1	2.47	2.44	2.36
13	Indiana	Trefoil	30.1	38.8	1.0	1.29	18.2	3.59	4.23	3.04
14	Iowa	Clover	30.4	38.2	0.2	1.26	18.7	1.88	3.08	0.67
15	Iowa	Clover	31.3	38.9	1.0	1.24	15.6	3.04	3.57	2.18
16	Iowa	Clover	32.9	42.9	0.4	1.30	18.0	1.71	3.89	1.61
17	Manitoba	Clover	32.9	40.0	4.2	1.22	18.4	1.37	2.08	2.32
18	Mississippi	Rattan Vine	24.9	34.0	1.3	1.37	15.2	ND	ND	1.40
19	Mississippi	Gallberry	28.6	38.0	2.2	1.33	18.2	ND	2.22	0.80
20	Mississippi	Soybean	30.5	40.0	0.8	1.31	19.7	1.87	1.82	2.67
21	Mississippi	Sourwood	27.7	39.0	2.4	1.41	16.2	ND	1.95	12.6
22	Montana	Alfalfa	28.9	34.2	0.9	1.18	17.5	ND	2.52	1.01
23	Montana	Alfalfa	33.6	41.4	0.7	1.23	14.1	ND	2.08	1.70
24	Montana	Knap-weed	29.2	36.5	4.9	1.25	16.9	2.41	3.93	3.18
25	Montana	Alfalfa Clover	30.6	38.3	4.7	1.25	16.8	ND	3.03	2.45
26	Nebraska	Alfalfa	32.0	38.7	0.8	1.21	16.2	2.42	2.00	1.05
27	Nebraska	Alfalfa	31.8	39.4	1.3	1.24	17.4	ND	4.07	3.05
28	New York	Clover	29.1	37.0	2.5	1.27	18.6	1.37	3.00	3.17
29	North Dakota	Sunflower	33.5	39.5	0.9	1.18	15.6	1.30	2.40	0.76
30	North Dakota	Clover	33.8	39.9	0.8	1.18	15.7	0.91	1.79	0.57
31	North Dakota	Alfalfa Clover	31.6	39.2	1.0	1.24	15.9	ND	4.20	1.29
32	Nuevo Leon	Orange	31.0	40.6	1.9	1.31	18.9	ND	3.81	ND
33	Ohio	Clover	31.2	38.8	0.6	1.24	17.3	3.03	4.35	4.03
34	Ohio	Locust Clover	24.4	43.9	2.6	1.80	16.9	3.40	4.74	5.79
35	Ohio	Clover	30.6	39.0	0.3	1.27	17.0	ND	3.13	ND
36	Saskatchewan	Canola	39.8	38.6	0.6	0.97	18.4	ND	ND	ND
37	Saskatchewan	Borage Canola	38.8	37.9	0.6	0.97	16.8	ND	2.34	1.56
38	South Carolina	Sourwood	26.1	41.7	1.8	1.60	18.2	ND	1.49	1.14
39	South Carolina	Tulip Poplar	31.6	38.9	3.1	1.23	17.8	ND	4.04	1.79
40	South Dakota	Clover	31.0	37.2	0.6	1.20	17.0	1.62	3.47	2.05
41	South Dakota	Buck-wheat	31.9	39.0	1.0	1.22	18.3	0.71	0.78	1.55
42	Texas	Cotton	35.3	37.2	1.4	1.05	18.8	ND	2.51	0.56
43	Vermont	Clover	28.7	37.5	1.7	1.31	19.4	1.37	2.84	6.59
44	Wisconsin	Clover	28.0	38.4	1.4	1.37	17.6	4.09	3.05	0.78

[a] Beekeeper's estimate.
[b] ND, not detected.

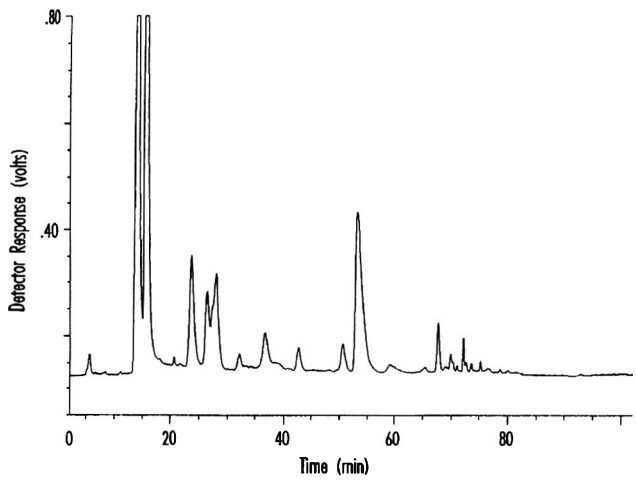

Figure 1. LC chromatogram of the carbohydrates in a pure honey (sample 30) after charcoal–Celite chromatography (method 1).

lyzed were similar, variations in the concentration of oligosaccharides were observed. Explanations for these differences include conditions in the honey (pH, presence of minerals, etc.) that could inhibit or favor the enzymatic formation of oligosaccharides; storage time of the honey; temperature of storage; and glucose, fructose, and sucrose concentrations in the original nectar. For example, Canola honey contains only small amounts of sucrose and erlose (25). This can be explained by the fact that Canola nectar has been shown to contain a very low concentration of sucrose (24). In addition, because erlose is formed by the transfer of an α-glucose moiety to sucrose by α-glucosidase, the low concentration of this carbohydrate in Canola honey can also be readily explained.

Acid hydrolysis of cane and beet sucrose is the main commercial method used to produce invert syrups (Alberta Sugar Company, personal communication). Oligosaccharides are synthesized also during this hydrolysis, but these reversion

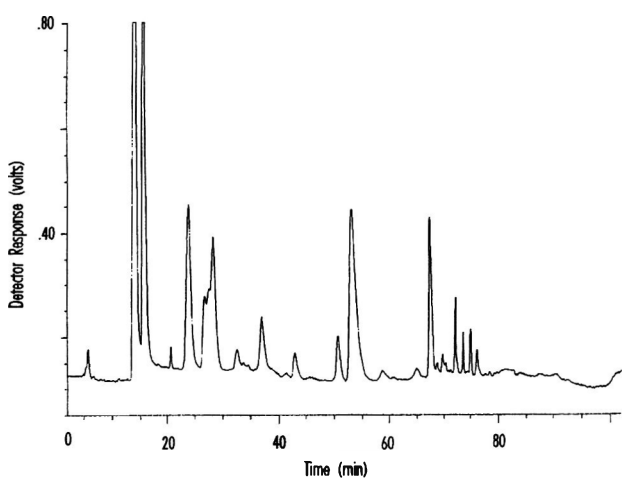

Figure 2. LC chromatogram of the carbohydrates in a pure honey (sample 35) after charcoal–Celite chromatography (method 1).

Table 4. Glucose, fructose, sucrose, and water contents of 6 invert syrups and 4 HFCS 55

Sweetener	Glucose, %	Fructose, %	Sucrose, %	Water, %
IS-C1	34.0	31.6	0.8	20.4
IS-C2	32.5	32.0	4.3	21.6
IS-C3	34.8	32.6	0.3	20.9
IS-C4	33.9	32.9	1.0	26.1
IS-C5	31.3	29.9	2.6	26.2
IS-E	37.9	37.2	1.9	19.9
HFCS1	33.4	43.8	—	20.9
HFCS2	33.7	47.1	—	21.2
HFCS3	35.6	41.8	—	21.4
HFCS4	33.8	47.3	—	23.4

compounds contain both α- and β-glucose and α- and β-fructose moieties due to the nonstereospecificity of this chemical reaction. The glucose, fructose, sucrose, and water contents of 6 invert syrups (from both cane and beet sucrose) are listed in Table 4. Figure 3 is an LC chromatogram of the oligosaccharide profile found in a chemically produced commercial invert syrup (IS-C1) after charcoal–Celite treatment. The peak at 26 min in this chromatogram is sucrose. Figure 4 is an LC chromatogram of the oligosaccharides in an enzymatically produced invert syrup (IS-E) after charcoal–Celite treatment. As is the case with any chromatographic method, there were some retention time shifts with these fingerprint peaks. However, the maximum retention time shifts observed for these compounds throughout this study were consistently <3%.

Detection of Invert Syrups in Honey

LC analysis (method 1) of a pure honey sample (Figure 1) intentionally adulterated with IS-C1 at a level of 10% (Figure 5) revealed that detection of this sweetener was possible by monitoring a peak in the 21 min region (peak I). The peak at

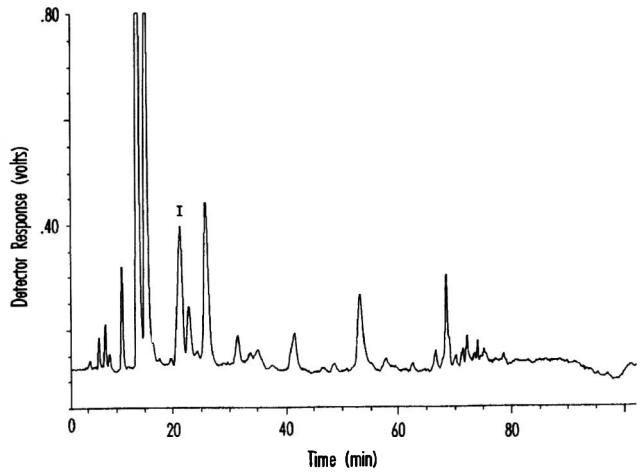

Figure 3. LC chromatogram of the carbohydrates in a chemically produced commercial invert syrup (IS-C1) after charcoal–Celite chromatography (method 1). Peak I is the fingerprint peak.

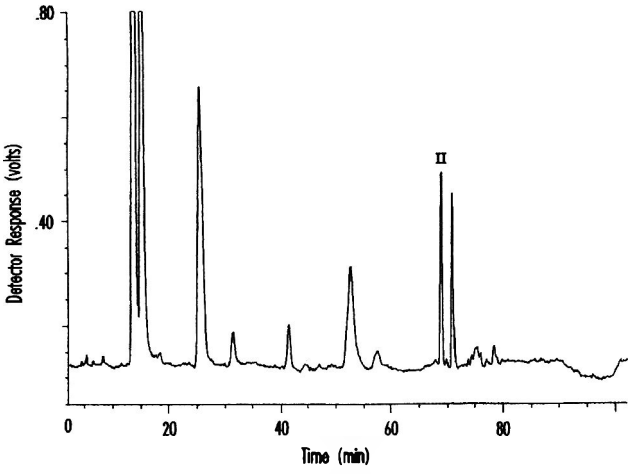

Figure 4. LC chromatogram of the carbohydrates in an enzymatically produced invert syrup (IS-E) after charcoal–Celite chromatography (method 1). Peak II is the fingerprint peak.

12 min in Figure 3 was not present in all the invert syrups tested and could not be used as a marker. The carbohydrate eluting at 21 min (not identified) could not be detected in 22 of the 44 pure honey samples analyzed and was present in low concentrations in the remaining samples, as shown in Table 3. The area of this peak in the pure samples ranged from 0 to 4.09 $\times 10^5$, with an average area of 1.13×10^5.

Table 5 lists the areas of peaks I, II, and III in 5 commercial chemically produced invert syrups, 1 enzymatically produced invert syrup, and 4 commercial high-fructose corn syrups. IS-C1 (from cane sucrose) and IS-C2 (from beet sucrose) were added to 26 of the honeys at a level of 10% (w/w) to determine the increase in area of peak I. IS-C1 was chosen because it was derived from cane and IS-C2 was chosen because it was one of the chemically produced beet invert syrups that had a low concentration of peak I. The addition of IS-C1 at a level of 10%

Table 5. Areas of marker peaks in 10 inexpensive sweeteners

Sample	Dilution factor	Peak I area, $\times 10^6$	Peak II area, $\times 10^6$	Peak III area, $\times 10^6$
IS-C1	10	2.58	—	—
IS-C2	10	1.04	—	—
IS-C3	10	1.32	—	—
IS-C4	10	2.72	—	—
IS-C5	10	0.98	—	—
IS-E	5	—	1.87	—
HFCS1	10	—	—	1.24
HFCS2	10	—	—	1.36
HFCS3	10	—	—	1.69
HFCS4	10	—	—	1.58

raised the area of peak I to an average of 2.24×10^6 with a range of 1.63 to 2.98×10^6 in the honeys. Addition of IS-C2 at a level of 10% raised the area of peak I to an average of 1.16×10^6 with a range of 0.90 to 1.65×10^6.

Enzymatically produced invert syrup (IS-E) was added to the pure honey shown in Figure 1 to levels of 10 and 20%. The LC chromatogram (method 1) of the honey with 10% IS-E is shown in Figure 6. The main problem with the detection of IS-E in honey was that there was no easily detectable peak in this syrup that was not present in most of the pure honey samples analyzed. Optimum results were obtained when the peak at 70 min (peak II) was monitored. This carbohydrate (not identified) was present in 39 of 44 pure honeys. The area of peak II in the pure honeys averaged 2.43×10^5 with a range of 0 to 4.74×10^5 as shown in Table 3. Addition of IS-E to a level of 10% in 26 honeys resulted in an increase in the area of peak II to an average of 1.05×10^6 with a range of 4.30 to 1.76×10^6. Addition of IS-E to a level of 20% resulted in an increase in the area of peak II to an average of 1.82×10^6 with a range of 1.23

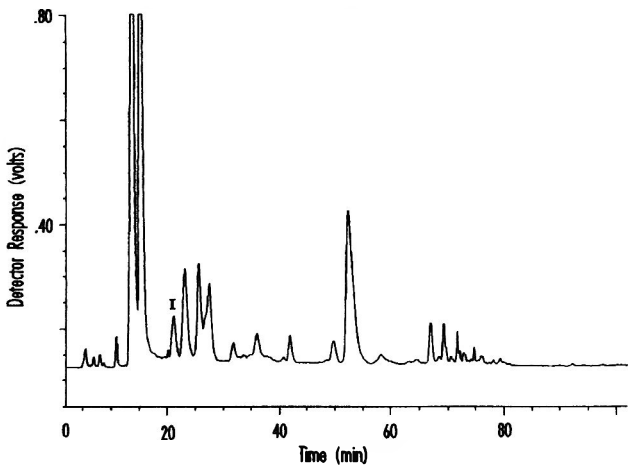

Figure 5. LC chromatogram of the carbohydrates in honey (sample 30) with 10% IS-C1 after charcoal–Celite chromatography (method 1). Peak I is the fingerprint for chemically produced invert syrups in honey.

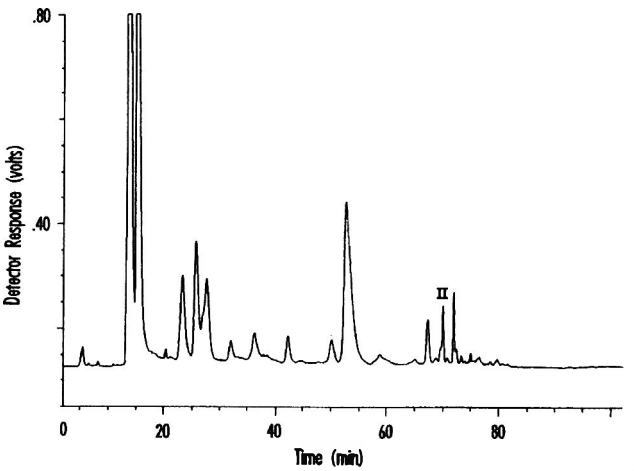

Figure 6. LC chromatogram of the carbohydrates in honey (sample 30) with 10% IS-E after charcoal–Celite chromatography (method 1). Peak II is the fingerprint for IS-E in honey.

to 2.38×10^6. These results indicated that detection of the addition of 20% enzymatically produced invert syrup to honey was feasible.

Detection of High-Fructose Corn Syrup in Honey

Attempts were made to use the same LC elution program for the detection of HFCS to detect invert syrups in honey. Unfortunately, under these LC conditions there were no fingerprint peaks in HFCS that distinguished this syrup from pure honeys at the 10% adulteration level. The LC program was altered to separate more effectively the dextrose polymers present in commercial HFCS. These changes included a final sodium acetate concentration of 250 mM, use of 1 analytical column, flow rate of 1.0 mL/min, and an increase in sample injection volume to 600 µL. By using this new LC method (method 2), the detection of 10% HFCS in the pure honeys was achieved. Figure 7 is an LC chromatogram of the carbohydrates in a representative pure honey (sample 30) obtained with the new program. The glucose, fructose, sucrose, and water contents of 4 commercial HFCS 55 (55% fructose) are listed in Table 4. Figure 8 is a chromatogram of HFCS1. HFCS 42 has been shown to have an identical oligosaccharide pattern (32). Figure 9 shows the pure honey (sample 30) with 10% HFCS1. This HFCS was chosen as the honey adulterant because it contained the lowest concentration of peak III (Table 5). The fingerprint oligosaccharide eluting at approximately 40.9 min (peak III) was used to detect the addition of HFCS. This carbohydrate was not detected in 4 of 44 of the pure honeys and was present in low concentrations in the remaining samples, with the exception of sample 21, as shown in Table 3. The average area of this peak in the pure honeys was 2.07×10^5 with a range of 0 to 12.6×10^5. After addition of HFCS1 to a level of 10%, the area of this peak increased to 1.97×10^6 with a range of 1.25 to 2.85×10^6. Although this carbohydrate was not identified, several glucose polymer standards were analyzed by using this LC

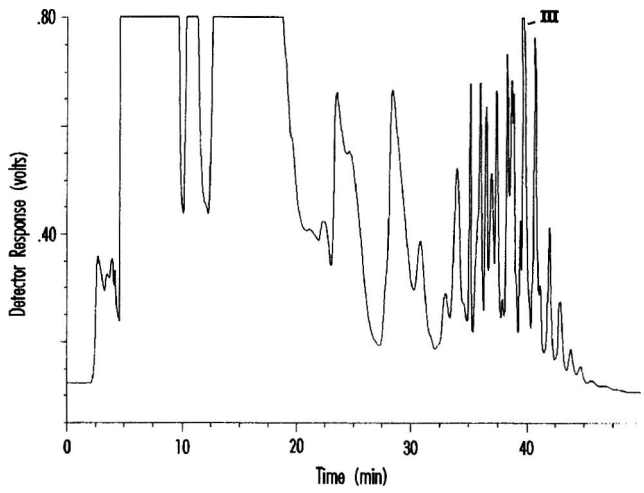

Figure 8. LC chromatogram of the carbohydrates in commercial HFCS1 after charcoal–Celite chromatography (method 2). Peak III is the fingerprint for HFCS in honey.

program and the retention time of the carbohydrate in peak III was identical to a branched DP6 standard.

Table 6 shows the reproducibility of retention times and peak areas in 6 honeys with 10% IS-C1 (peak I), 20% IS-E (peak II), and 10% HFCS1 (peak III). The average retention time and area for peak I in the honey samples with 10% IS-C1 were 21.1 min and 2.24×10^6, respectively, with an average standard deviation of 0.08 and 0.17×10^6, respectively. The average retention time and area for peak II in the honey samples with 20% IS-E were 70.2 min and 1.82×10^6, respectively, with an average standard deviation of 0.16 and 0.14×10^6, respectively. The average retention time and area for peak III in the honey samples with 10% HFCS1 were 40.9 min and 1.97

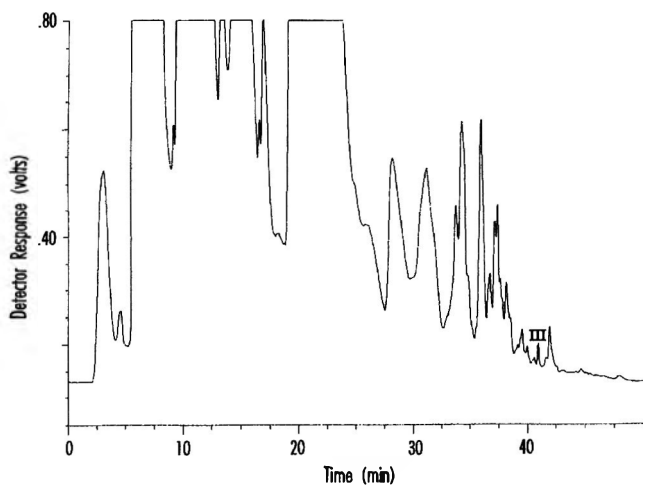

Figure 7. LC chromatogram of the carbohydrates in a pure honey (sample 30) after charcoal–Celite chromatography (method 2). Peak III is the fingerprint for HFCS in honey.

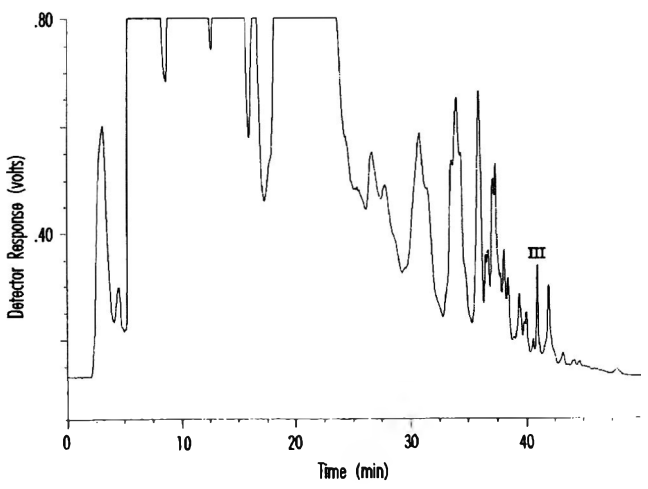

Figure 9. LC chromatogram of the carbohydrates in honey (sample 30) with 10% HFCS1 after charcoal–Celite chromatography (method 2). Peak III is the fingerprint for HFCS in honey.

Table 6. Reproducibility of retention times (t_R) and peak areas of 6 honeys with 10% IS-C (peak I), 20% IS-E (peak II), and 10% HFCS (peak III)

	Peak I		Peak II		Peak III	
Honey sample	t_R, min	Area $\times 10^6$	t_R, min	Area $\times 10^6$	t_R, min	Area $\times 10^6$
6	20.97	1.68	69.86	1.43	41.08	1.78
6	21.00	1.63	69.26	1.68	40.83	1.84
10	21.13	2.98	69.93	1.54	41.03	2.33
10	21.18	2.82	69.69	1.77	41.11	2.48
11	21.16	2.20	69.80	2.13	40.99	2.60
11	21.36	2.38	70.14	1.79	40.97	2.36
15	20.98	2.47	69.71	1.84	40.29	1.33
15	21.10	2.17	69.28	2.06	40.39	1.45
34	21.26	2.62	70.41	1.85	40.49	2.23
34	21.15	2.33	70.33	1.89	40.45	1.99
36	21.08	2.20	70.24	2.12	40.85	2.67
36	21.23	2.56	70.58	2.14	40.91	2.85

$\times 10^6$, respectively, with an average standard deviation of 0.14 and 0.16×10^6, respectively.

Conclusions

Honey, chemically and enzymatically produced invert syrups, and high-fructose corn syrup each contain a complex mixture of oligosaccharides that are synthesized during the production of these foods. The presence of fingerprint oligosaccharides in inexpensive sweeteners can be used to detect their illegal addition to pure foods such as honey. The use of LC-PAD to determine honey authenticity looks promising, although the need to analyze more pure honey samples to establish baseline data is recognized. Methodology developed here could be extremely useful in the analysis of honey for the presence of invert syrups and HFCS.

Acknowledgments

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FOOD COMPOSITION AND ADDITIVES

Enzymatic/Chemical Analysis of Dietary Fiber

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The Uppsala methodology for rapid analysis and characterization of total dietary fiber, defined as the sum of dietary fiber polysaccharides (DFP) and Klason lignin, was studied. A sugar- and starch-free residue was prepared by treatment with a thermostable amylase and amyloglucosidase. Neutral DFP residues were quantified by gas chromatography as alditol acetates after acid hydrolysis of this residue, and the acid-insoluble fraction, Klason lignin, was determined gravimetrically. Uronic acid residues were quantified by decarboxylation of the original sample. The efficacy of the Uppsala methodology was tested with foods varying in fiber content and composition, including heat-treated samples. The present method allowed the analysis of up to 40 samples per week. It had good repeatability and coefficients of variation of 3–5% for the main fiber components. Fiber contents determined with the method were higher than those determined with a similar method that excludes Klason lignin and starch resistant to amylases but soluble in dimethyl sulfoxide and lower than those determined with an enzymatic/gravimetric method. Important aspects of fiber analysis, like enzyme purity and the recovery of soluble fiber on ethanol precipitation, also were investigated.

An understanding of the nutritional effects of dietary fiber has been hampered considerably by the lack of an appropriate definition of fiber and by inadequate analysis methods. On the basis of physiological criteria, Trowell (1) defined dietary fiber as "the remnants of the plant cell wall that are not hydrolyzed by the alimentary enzymes of man," and this definition was later simplified and expanded to include "the plant polysaccharides and lignin which are resistant to hydrolysis by the enzymes of man" (2). In 1979, we proposed that dietary fiber could be defined as the sum of nonstarch polysaccharides (NSP) and Klason lignin, and in conjunction with this chemical definition, we published the first gas chromatographic method for the analysis and characterization of dietary fibers (3). Because nonstarch polysaccharides contain some enzyme-resistant starch, the term dietary fiber polysaccharides (DFP) is preferred. Because this analytical procedure includes starch resistant to α-amylases and lignin, it conforms well to the original definitions by Trowell and co-workers (1, 2). This resistant starch is generally only part of the physiologically resistant starch not digested in the small intestine (4). Our original dietary fiber method, first presented in 1978 in Cambridge at a European Economic Community meeting, has now gained merit as a citation classic (5).

The Uppsala method is based on the prior removal of free sugars and starch, including treatment with a thermostable α-amylase, followed by acid hydrolysis and determination of neutral polysaccharide residues as alditol acetates by gas-liquid chromatography (GLC). Klason lignin, the noncarbohydrate fraction of dietary fiber, is determined gravimetrically as the acid-insoluble material and uronic acid residues by decarboxylation. Recently, the method was improved to essentially a 1-tube procedure with a consequent increase in speed of analysis (6).

In addition to this method, 2 other methods, the UK method and the AOAC method, are often used in the analysis of dietary fiber (7). The former, which is based on the work of Southgate (8), is today, after several modifications, relatively similar to our procedure in that DFP residues are determined by GLC. Thus, for example, correction factors were recently included in the determination of neutral DFP residues (9), and more recently, α-amylase (Termamyl) was also included (10). However, the UK method does not include any measure of lignin and has an extraction step for the removal of starch not hydrolyzed by α-amylases but soluble in dimethyl sulfoxide or aqueous alkali, the so-called enzyme-resistant starch. The Lund procedure, on which the AOAC method (11) is, to a great extent, based, is similar to the Uppsala methodology in that it includes the degradation of starch by Termamyl (12). However, this step is followed by the enzymatic degradation of protein and the gravimetric determination of the resultant dietary fiber residue. Although designed to be fast and simple, this method is rather

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laborious because of the need to correct the fiber residue for contamination by protein and ash. When determination of uronic acids is performed by colorimetry instead of decarboxylation, the difference in analytical capacities of the Lund and Uppsala methodologies becomes smaller. In this paper, the improved original method is compared with the UK and Lund methods for the determination of dietary fiber in various food samples. Some of the results were recently reported (13). We also discuss here some pertinent problems with fiber analysis, including definition of dietary fiber, recovery of soluble fibers during ethanol precipitation, enzyme purity, and determination of resistant starch.

Materials and Methods

Samples

(**a**) *Cornflakes.*—Commercial (Kelloggs, S-102 62 Stockholm, Sweden).

(**b**) *Dry green peas.*—Commercial (Nutana, DK-4632 Bjaershov, Denmark).

(**c**) *Yellow onions.*—Bought in a local store and cut, after removal of the skin, into 5 mm-thick slices, which were freeze-dried.

(**d**) *Rye crisp bread.*—Commercial (Wasabröd AB, S-682 82 Filipstad, Sweden), containing whole rye meal and wheat bran, with a declared dietary fiber content of 26%.

(**e**) *Sugar beet fiber.*—Commercial (Fibrex, Swedish Sugar Co., S-200 10 Malmö, Sweden).

(**f**) *Wheat flour rolls.*—Baked from high-quality flour according to the following recipe: 1800 g flour, 1000 g water, 100 g yeast, 30 g salt, 30 g sucrose, and 30 g margarine. The rolls were baked for 12 min at 250°C and, when cool, were separated by hand into crust and crumb fractions. On a dry-matter basis, the rolls contained 58% crumb and 42% crust.

(**g**) *Whole-fat soybeans.*—Imported by Västsvenska lantmän (S-531 87 Lidköping, Sweden).

All samples (about 600 g of each) were ground on a Tecator Cemotec 1090 sample mill at a setting of 1.5 and further on a Tecator Cyclotec 1093 sample mill to pass a 0.5 mm screen.

Enzymatic/Chemical Methods for Determination of Dietary Fiber

All samples were analyzed at least in duplicate. Dietary fiber was determined by the Lund method as described by Asp et al. (12). A 0.5–1 g sample was incubated at pH 6.0 for 15 min at 100°C with α-amylase (Termamyl) and allowed to cool. The pH was adjusted to 1.5, and the sample was incubated with pepsin for 60 min at 40°C; pH was adjusted to 6.8, and the sample was treated further with pancreatin for 60 min at 40°C. After the sample was cooled, the pH was adjusted to 4.5, and the soluble fiber precipitated during 1 h after addition of 4 volumes of 95% ethanol. Total fiber was recovered by filtration, and the residue was corrected for the amount of ash and protein.

Dietary fiber also was determined according to the UK method (9). A 200 mg dry sample was boiled in dimethyl sulfoxide for 1 h to disperse all starch. Then the sample was treated with α-amylase (pancreatin) and pullulanase at 42°C for 16 h. The soluble fiber was precipitated with 80% ethanol (v/v) for 1 h at room temperature and then centrifuged. The insoluble residue was washed with ethanol and then acetone, dried, and then subjected to sequential acid hydrolysis by treatment with (*1*) 12M sulfuric acid for 1 h at 35°C and (*2*) 1M sulfuric acid for 2 h at 100°C. Neutral sugars in the hydrolysate were quantified by GLC of alditol acetates and colorimetry of uronic acids.

A modification of the method developed in Uppsala in 1979 was used for determination of total dietary fiber (6, 13). Representative samples were ground to pass a 0.5 mm screen or freeze-dried before grinding if the water content exceeded 15%. If more than 6% fat was present in the sample, ultrasonic pre-extraction with petroleum ether and air-drying was recommended. The sample (250–500 mg of dry matter) was then incubated with α-amylase (Termamyl) for 1 h in a boiling water bath at pH 5.0 and further for 4 h at 60°C with an amyloglucosidase. Next, soluble fibers were precipitated with 80% (v/v) ethanol for 1 h at 4°C. The insoluble fraction was isolated by centrifugation, washed with 80% ethanol and acetone, dried at 40°C, and subjected to sequential acid hydrolysis with (*1*) 12M sulfuric acid (1 h, 30°C) and (*2*) 0.41M sulfuric acid (1 h, 125°C) by autoclaving.

The hot hydrolysate was filtered through a glass filter, and the amount of insoluble residue was determined gravimetrically as Klason lignin (after ashing). The neutral monosaccharides in the filtrate were reduced and acetylated with 1-methylimidazole as catalyst, and the formed alditol acetates were quantified by GLC on a DB-225 capillary column with correction factors for the individual sugars. These factors account for losses during hydrolysis and derivatization and for the response on the GLC. The values for monosaccharides were then expressed as polysaccharide residues (anhydro-sugars) by multiplying the amounts of pentoses and deoxypentoses with 0.88 and of hexoses with 0.90.

The uronic acid content was determined by refluxing an aliquot of the original dry sample (50–100 mg, containing 1–10 mg of uronic acids) with hydriodic acid in a small round-bottomed flask connected to the decarboxylation equipment. Released CO_2 was trapped in 15 mL of thermostated (25°C) 0.02M sodium hydroxide; the changes in conductance were registered by a potentiometric recorder. The uronic acid content is directly proportional to the deflection obtained on extrapolating a tangent to the conductance curve back to zero time (3). D-Galacturonic acid monohydrate was used as a standard, and results were expressed as polysaccharide residues (anhydro-sugars). Dietary fiber was then calculated as the sum of neutral polysaccharide residues, uronic acid residues, and Klason lignin.

Analysis of Polysaccharides Soluble in 80% Aqueous Ethanol

The 80% ethanolic supernatants, which were obtained during isolation of fiber residues of the respective foods, were concentrated to dryness by rotary evaporation (<40°C). The resulting residue was taken up in water (50 mL), and the solution was

defatted by extraction with chloroform (3 × 20 mL). Next, the aqueous layer was diluted to 100.0 mL with water and centrifuged (2000 × *g*; 10 min); the supernatant was decanted, and 2 aliquots (5.0 mL) of this solution were concentrated to dryness. The syrup obtained (always less than 150 mg) was dissolved in 1.0 mL of water containing 2-methyl-1,1,1-trichloro-2-propanol (50 mg/L) as a preservative (Fluka, CH-9470 Buchs) and then subjected to preparative gel filtration on Biogel P-2 (BioRad) in this solvent. The column (90 × 2.5 cm) was calibrated with a maltodextrin mixture from our laboratory. A column flow rate of 15.0 mL/h was used, and peaks eluted were detected by their UV (at 280 nm) and/or refractometer responses.

By comparison with the elution pattern of a maltodextrin standard mixture, fractions from different foods containing polysaccharides (degree of polymerization, >10) were collected (Figure 1). Each fraction was concentrated to dryness, and aqueous solutions of *myo*-inositol (0.50 mg in 0.50 mL) and trifluoroacetic acid (2M, 0.50 mL) were added to the test tubes, the test tubes were capped, and the mixture was hydrolyzed at 121°C for 90 min. Monosaccharides in the resulting hydrolyzate were quantified in duplicate as alditol acetates by GLC according to the Uppsala method.

Determination of Enzyme Resistant Starch

The analytical procedure was described previously (14). In brief, starch was removed from original food samples (90.0–100.0 mg) by 2 incubations at 96°C for 30 min with Termamyl 120L in acetate buffer (pH 5, 0.1M), followed by 1 incubation with amyloglucosidase at 60°C overnight. Resistant starch in the resulting insoluble residue was solubilized by mixing with 2M aqueous potassium hydroxide for 30 min; the mixture was neutralized by adding 2M HCl and acetate buffer (0.4M, pH 4.8) and then hydrolyzed with amyloglucosidase overnight. Glucose released was determined by the glucose oxidase method.

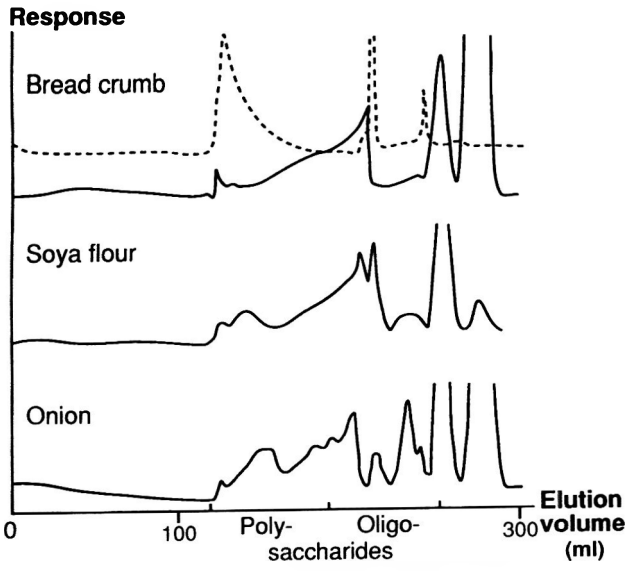

Figure 1. Gel filtration on Biogel P-2 of carbohydrates in supernatants obtained during the 80% ethanol precipitation of soluble fibers by the Uppsala method. The refractometer response (solid line) and UV response at 280 nm (dotted line) were used in comparison with a maltodextrin standard to determine elution volumes for poly- and oligosaccharides.

Results and Discussion

Sample Preparation

It is important that samples are ground or homogenized to a small and consistent size prior to analysis, and the type of mill used in this study is recommended. Homogenization followed by freeze-drying and grinding may be preferable for samples of low dry matter. With samples, such as the soybean and rye crisp studied here, that have a fat content over 6%, it is advisable to pre-extract with petroleum ether. However, the fat does not need to be removed completely, and one extraction after dispersion in petroleum ether should be sufficient. This step can be done after weighing of the sample for fiber analysis and prior to starch removal to avoid reweighing or sample transfer after defatting. In this study it was found, however, that defatting of the soybean had no effect on the analyzed content of neutral DFP residues but reduced ($P < 0.05$) the Klason lignin content from 1.4% (SD, 0.19; $n = 3$) to 0.8% (SD, 0.09; $n = 3$).

Starch Hydrolysis

If incomplete hydrolysis of starch or incomplete recovery of dietary fiber occurs, it is generally because of poor enzyme quality, often amyloglucosidase. For example, when the amyloglucosidase used here was used in the analysis of a pure mixed-linked β-glucan (15) at the same enzyme:sample ratio as used in the fiber analysis, recovery of the β-glucan was 100%. When tested at a 10-fold higher enzyme concentration, recovery was 93%, indicating an insignificant β-glucanase activity. However, many commercial amyloglucosidases, including some used in other fiber analysis methods, had unacceptably high β-glucanase activity, and this varied considerably between enzyme preparation and batch. Enzyme batches should therefore be tested for absence of mixed-linked β-glucanase and pentosanase activities (3). These tests should be carried out under conditions compatible with the fiber analysis; for example the α-amylase (Termamyl) used here may have contaminating activities, but these are inactive at the high temperatures used during fiber analysis. Thus, it is desirable that, after addition of this enzyme, the samples are immersed quickly in the boiling-water bath. Also, it is necessary to take particular care to thoroughly disperse starch-rich samples, like white flour, during the initial Termamyl treatment, because a pellet partly resisting amylolytic digestion may otherwise be formed in the bottom of the tube.

The use of the thermostable α-amylase allows simultaneous gelatinization and degradation of the starch and thus prevents the formation of resistant starch. This enzyme also can degrade starch–lipid complexes (16). Under the conditions used in this study, the starch in 500 mg of the relatively starch-rich bread

crumb was completely removed after treatment with Termamyl and 2 h of incubation with amyloglucosidase at 60°C, indicating that the 4 h incubation time used in this method is more than adequate.

Recovery of Soluble Fiber

In many methods developed for determination of dietary fiber, water-soluble polysaccharides are precipitated with 80% aqueous ethanol. To investigate the effectiveness of this precipitation method, the content and composition of neutral nonstarch polysaccharides remaining in 80% ethanolic solution were determined. After subsequent gel filtration on Biogel P-2 (Figure 1), it was possible, by comparison with a maltodextrin standard, to isolate a fraction containing polysaccharides with a degree of polymerization greater than 10. Gel filtration was preferred to dialysis and freeze-drying as a means of isolating the polysaccharides, because in this way losses of polysaccharides during dialysis are avoided. The fractions isolated from samples in the present study were analyzed for neutral DFP contents.

The analysis established (Table 1) that a small and varying percentage (0.7–5.6%) of the total fiber content of the samples was lost because of the different solubilities of polysaccharides in 80% ethanol. The losses were highest for the heat-treated bread samples. Although different individual sugar residues were present in all fractions analyzed, some conclusions can be drawn regarding the type of polysaccharides present. It is well-known that highly branched polysaccharides often are more soluble in 80% ethanol (17). The arabinose residues from the sugar beet fiber sample were thus very likely the result of incomplete precipitation of a highly branched arabinan (18). In the onion sample, the predominance of glucose and mannose residues in the polysaccharides not precipitated by 80% ethanol presumably originated, at least partly, from low-molecular-weight fructans (19). During acid hydrolysis significant amounts of fructose residues are degraded (20), but some fructose may survive and be transformed to a mixture of glucitol and mannitol by the borohydride reduction in the analysis. The relatively high amount of mannose residues from the bread samples was probably caused by the presence of yeast mannans (14). The glucose residues from the same samples indicated some fragmentation of nonstarch glucans and/or chemical modification of starch, occurring as a result of heat treatment. We have previously shown that fragmentation of starch during baking gives rise to fragments containing β-1,6-anhydroglucopyranose as end residues, which may react further by transglycosidation to form branched nonstarch structures (21). Recovery of soluble fiber was not improved by prolonging the precipitation time at 4°C from 1 to 8 h.

Hydrolysis, Reduction, and Acetylation

Samples must be dry and finely dispersed prior to treatment with 12M sulfuric acid. A wet pellet may be incompletely dispersed in the sulfuric acid and therefore incompletely hydrolyzed. Incomplete hydrolysis will primarily result in a low value for glucose residues (cellulose) and a high Klason lignin content. When hydrolysis conditions are optimized, some degradation of DFP residues, particularly pentoses, is inevitable. Such hydrolysis losses necessitate the use of correction factors (22).

Incomplete reduction of monosaccharides can occur if the pH of the reaction mixture is too low or if deteriorated potassium borohydride is used. This condition is indicated by the appearance of ghost peaks on the GLC. The alditols are acetylated by using 1-methylimidazole as catalyst (23), thus essentially simplifying this procedure to a 1-tube step. We recently showed that the neutralization and reduction steps may be omitted by direct acetylation, with quantification of the multiple peaks of the individual sugars by multivariate calibration (24). So far, this time-saving alternative has been applied only to a few plant-based samples; however, results are in good agreement with the present method.

Quantification of DFP Residues and Klason Lignin

GLC is used to quantify neutral DFP residues, but liquid chromatography is also a possible alternative (25). Instead of DB-225, other equivalent columns such as Cp-Sil 88 may be used in the GLC analysis (23).

Correction factors for quantification depend on the hydrolysis, derivatization, and GLC procedures used. These factors should be updated regularly and can be checked by including a suitable fiber standard; we routinely include a purified wheat

Table 1. Neutral dietary fiber polysaccharide (DFP) residues not recovered on precipitation of soluble fiber with 80% ethanol in samples analyzed by the Uppsala methodology

	DFP residues not recovered, % of total fiber content						
Sample	Rhamnose	Arabinose	Xylose	Mannose	Galactose	Glucose	Total
Corn flakes	0.2	0.2	0.2	0.5	0.5	1.0	2 6
Bread crust	0.2	0.2	0.2	0.7	0.5	3.8	5 6
Bread crumb	0.2	0.2	0.2	1.1	0.2	1.9	3.8
Rye crisp	0.1	0.1	0.1	0.3	0.1	1.1	1.8
Green peas	0.1	0.1	0.1	0.1	Trace	0.3	0.7
Soybean	0.2	0.1	0.2	0.3	0.3	0.4	1 5
Deskinned onion	0.5	0.1	0.1	0.3	0.2	1.1	1 9
Sugar beet fiber	0.1	0.5	0.1	0.1	0.1	0.1	1 0

fiber sample (Tricum Ave, S-263 21 Höganäs) to check recovery and repeatability of individual sugar figures.

If Klason lignin constitutes less than 1% of a sample, then the gravimetric determination of a residue of <5 mg will require skill and appropriate equipment. Care should also be taken to ensure that the Klason lignin residue is washed adequately during recovery; otherwise some degradation and losses may occur during drying.

Quantification of Uronic Acids

The decarboxylation method used here is essentially free from interference from other food components such as neutral sugars and proteins (3), which is often a problem with colorimetric methods. The response obtained is also independent of the type of uronic acid present and is linear over the conductivity range used. Although the analysis of each sample for uronic acids takes about 45 min, this method is not very time demanding and can be run parallel to the determination of the other fiber components. As an alternative, a colorimetric method for analysis of uronic acids (26) has been used in further studies because such a method is more available for most laboratories. The analysis, although probably not as specific as the decarboxylation method, can be done conveniently on the polysaccharide hydrolysate.

Fiber Analysis with the Uppsala Methodology

In this study 8 samples varying considerably in type and content of dietary fibers were analyzed. The rye crisp and soybeans were pre-extracted with petroleum ether to remove fats. The contents of dietary fiber (DFP residues + Klason lignin) ranged from 2.7% in cornflakes to 64.7% in sugar beet pulp (Table 2). Only small amounts of Klason lignin were present in most samples, but the rye crisp, which contained wheat bran, had 2.1%. Generally, low levels of rhamnose, fucose, and ribose residues were detected, whereas the content and relative composition of the DFP fraction varied considerably between samples. Glucose residues, mainly from cellulose, were predominant in most samples, with significant levels of pentosans (arabinose + xylose residues) in the cereal-based samples and of pectic polysaccharides (arabinose + galactose + uronic acid residues) in the other samples.

The mean coefficient of variation of independent triplicate analyses for the main DFP residues was 3–5%, indicating the good repeatability of the method. This variation tended to be lower in the samples with higher contents of the particular DFP residue. The corresponding variation in Klason lignin (11–31%) was understandably higher for the samples with low lignin content.

The starch-containing samples were analyzed for resistant starch, and cornflakes, bread crust, and bread crumb were found to contain significant amounts, a consequence of heating during preparation of these foods. However, the peas, which had not been heat treated, also contained some resistant starch. The range of the coefficient of variation for this analysis was also acceptable (5–13%), considering the low levels present in the samples. This method for the determination of resistant starch (14) is applied on original samples and not on isolated fiber fractions, as is the case for some other methods (27, 28). The present procedure thus diminishes the risk of forming resistant starch during analysis. It also avoids the use of dimethyl sulfoxide, which is a rather hazardous chemical and a solvent for some noncellulose polysaccharides (29, 30) and therefore could partly hinder precipitation of soluble fibers with ethanol.

Comparison of the Uppsala, UK, and Lund Methodologies

Total dietary fiber contents as determined by our method were compared with those obtained with the UK (9) and Lund (12) methods (Table 3). The last 2 analysis procedures were kindly carried out by The Rowett Research Institute, Aberdeen, Scotland (UK), and AnalyCen AB, Lidköping, Sweden (Lund), in laboratories that routinely use these methods. As expected, results from the UK method were on average lower (–6.2%; range, –60.3% to +3.6%) than those from the Uppsala method-

Table 2. Content of dietary fiber polysaccharide (DFP) residues, Klason lignin, and resistant starch in analyzed samples[a]

	DFP residues, % of dry matter										
Sample	Rhamnose	Fucose	Ribose	Arabinose	Xylose	Mannose	Galactose	Glucose	Uronic acids	Klason lignin	Resistant starch
Corn flakes	Trace	Trace	Trace	0.15	0.19	0.08	0.04	1.93	0.05	0.29	1.50
Bread crust	Trace	Trace	Trace	0.67	1.12	0.23	0.23	1.10	0.03	0.33	0.50
Bread crumb	Trace	Trace	Trace	0.63	1.04	0.26	0.24	1.38	0.03	0.25	0.80
Rye crisp	0.12	Trace	Trace	4.72	8.73	0.59	0.61	5.65	0.68	2.08	0.20
Green peas	0.36	0.06	0.09	2.56	1.33	0.21	0.86	6.66	2.01	0.33	0.30
Soybean	0.40	0.27	0.12	1.96	1.16	0.98	4.25	4.23	2.61	0.77	ND
Deskinned onion	0.38	0.09	Trace	0.42	0.58	0.37	5.35	5.74	6.19	0.96	ND
Sugar beet fiber	1.92	0.09	Trace	18.03	1.53	1.12	5.31	18.33	17.01	1.32	ND

[a] Values are averages of 3 analyses of individual components; ND, not determined.

ology. After adding the contents of Klason lignin and resistant starch, which are not routinely included in the UK method, the results became more similar.

The enzymatic/gravimetric Lund method, including correction for residual protein and ash, gave higher figures than the Uppsala procedure (Table 3). This discrepancy was not reduced by defatting of the samples prior to analysis by the Lund method. Using enzymatic/gravimetric methods, Marlett and co-workers showed a 14–18% higher fiber content in certain samples (31, 32). However, another study (33) showed good agreement between the Uppsala and Lund methodologies.

Contents of DFP residues were similar when analyzed by the Uppsala and UK methodologies. First-order regression of the data gave coefficients of determination approaching unity and intercepts close to the origin for all major neutral DFP residues (Table 4). For mannose residues, which were a relatively minor constituent, the Uppsala methodology gave slightly higher figures, as indicated by a coefficient of regression of less than 1, whereas the UK method gave higher figures for uronic acids. With the UK method, the regression line did not pass through the origin, presumably due to the fact that the UK method uses a colorimetric method whereas the Uppsala methodology uses a decarboxylation method for analysis. On the other hand, it is important to remember that these comparisons of the 3 dietary fiber methods are based on results from single laboratories only.

Conclusion

The Uppsala methodology for analysis of dietary fiber has been in use for 15 years. It has been applied to many types of samples, including human foods, animal feeds, and digesta and feces, and has proven to be reproducible, adaptable, robust, and accurate. Using the present improved procedure, a skilled analyst can run over 40 samples per week. It can be adapted easily to separately analyze soluble and insoluble fiber fractions (3). In this case the soluble fibers are separated by centrifugation after starch degradation and recovered by ethanol precipitation or dialysis and freeze-drying. The yield of soluble fibers, however, is very much dependent on extraction conditions used

Table 3. Content of dietary fiber determined by the Uppsala, Lund, and UK methodologies

Product	Dietary fiber content, % of dry matter		
	Uppsala	Lund	UK[a]
Corn flakes	2.7	4.5	0.8
Bread crust	3.7	4.6	2.7
Bread crumb	3.8	4.7	2.7
Rye crisp	23.2	27.0	20.0
Green peas	14.5	15.6	12.8
Soybean	16.8	18.6	15.1
Deskinned onion	20.1	22.6	19.2
Sugar beet fiber	64.7	76.5	67.0

[a] Measured as the sum of nonstarch polysaccharides

Table 4. First-order regression analysis between individual dietary fiber (DFP) residues analyzed by the Uppsala and UK methods for dietary fiber

DFP residue	Intercept	Coefficient of regression	Coefficient of determination, r^2
Arabinose	0.00	1.04	1.00
Xylose	0.00	0.98	1.00
Mannose	–0.04	0.73	0.96
Galactose	–0.04	0.91	1.00
Glucose[a]	–0.01	0.98	1.00
Uronic acids	–0.34	1.26	0.99

[a] The contents of resistant starch (Table 2) were added to the nonstarch glucose residue contents obtained with the UK method.

(34). At present, the Uppsala method, using a colorimetric assay instead of decarboxylation for uronic acid determination, is being evaluated in a collaborative AOAC study, with O. Theander as an associated referee.

There is still some controversy regarding which components should be included in the fiber complex, particularly regarding lignin and the starch that escapes enzymatic hydrolysis in the small intestine (35, 36). Lignin is closely associated, both physically and chemically, to the fiber polysaccharides in the plant cell walls and strongly influences the physicochemical properties of the fiber carbohydrates. The hydrophobic, phenolic polymer lignin per se also has some of the physiological properties that have induced so much interest in dietary fiber, including the binding of lipophilic components and the ability to increase fecal bulk (37, 38). The Klason lignin fraction isolated in the Uppsala methodology contains, in addition to lignin, other components such as cutins, tannins, and Maillard products (originating from thermal processing) (22). These components are also closely associated with the fiber complex and pass through the small intestine unabsorbed. Thus, their inclusion may be considered an advantage of this method, as is the fact that this fraction is obtained during preparation of the neutral DFP residues, thus making a separate analysis step unnecessary.

Some dietary starch passes through the small intestine and, like some fibers, is degraded or partly degraded in the hind gut. This includes analytically "enzyme-resistant" starch (27, 39), starch modified by chemical or heat treatment (14, 21), and other starch that, for various reasons, is not hydrolyzed in vivo by the enzymes present. Starch degradation will differ among individuals and will be influenced, for example, by eating habits, and thus cannot be estimated easily in the laboratory. However, the analytically enzyme-resistant and modified starch will always escape enzymatic hydrolysis. Further, it will undoubtedly function similar to other undigestible carbohydrates such as fiber polysaccharides. For example, they have been shown to reduce diverticulosis in rats (40). Thus, we consider that Klason lignin should be included in the dietary fiber complex, and like other widely used methods including the AOAC and Lund procedures, have developed our analytical scheme accordingly. That analytically enzyme-resistant starch is also included

in the fiber fraction conforms with the physiological definition of dietary fiber (2). Thus, like other researchers in this field (36), we do not consider at this time that special steps should be included in the analytical procedure for the removal of this component. Those particularly interested in starch resistant to amylases prior to extraction in certain solvents can analyze this component separately. However, whether certain other dietary components, including fructans, polysaccharides soluble in 80% ethanol, and various oligosaccharides, should be classified and analyzed as dietary fiber deserves further discussion.

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RESIDUES AND TRACE ELEMENTS

Liquid Chromatographic Fluorescence Method for the Determination of Thiabendazole Residues in Green Bananas and Banana Pulp

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A novel liquid chromatographic method was developed for the determination of thiabendazole (TBZ) residues in whole green bananas and ripe banana pulp. TBZ is extracted from the banana matrix with ethyl acetate, followed by cleanup of extract on a cation-exchange, solid-phase extraction column. The extract is analyzed for TBZ residues by column liquid chromatography using a cation-exchange column with fluorescence detection. Recoveries of TBZ from whole green bananas fortified with TBZ at 0.05–10 ppm and from ripe banana pulp fortified with TBZ at 0.01–2 ppm averaged 93 and 95%, respectively. The following method for monitoring TBZ residues in whole green bananas and ripe banana pulp is simple, rapid, and sensitive.

Discovered in 1961 by scientists at Merck Sharp & Dohme Research Laboratories and the Merck Institute for Therapeutic Research (1, 2), thiabendazole (TBZ; 2-(4-thiazolyl)-1*H*-benzimidazole; CAS No. 148-79-8) has been used widely as a broad-spectrum anthelmintic agent for domestic animals, including cattle, sheep, goats, horses, swine, dogs, and poultry (1, 3, 4) and as a pre- or postharvest systemic fungicide for the control of plant diseases for a wide variety of fruit and vegetables and other field crops (5–7). At present, one of the most important postharvest crop applications of TBZ is for protection against crown rot of bananas and plantains. TBZ (commonly applied as mist spray) is effective in eradicating pathogens such as *Fusarium roseum, Colletotrichum musae, Verticillium theobromae, Thielaviopsis paradoxa (Ceratocystis paradoxa), Botryodiplodia theobromae, Deightoniella torulosa,* and *Nigrospora* spp. (7, 8).

TBZ can be determined by column liquid chromatography (LC) with either UV or fluorescence detection. Because the reported pK_a values for TBZ are 2.5 and 4.7 (9), TBZ can be converted under acidic conditions to the cationic form, which is more water soluble than its neutral counterpart. Numerous techniques for the quantitative analysis of TBZ in various crops involve spectrophotometry and chromatography (9–20). In general, the recently published methods all involve liquid–liquid partitioning between organic solvents and acidic or alkaline solutions for the cleanup of TBZ extracts, followed by LC analysis with either UV or fluorescence detection. Most methods use either silica-based C_8 or C_{18} (octadecyl silica, ODS) columns, although some use bare silica, aminopropyl silica, and even porous polystyrene polymer containing CH_2OH groups as stationary phases. Surprisingly, very few methods use solid-phase extraction (SPE) for extract purification and concentration, and none take advantage of the ionic nature of TBZ by applying SPE and column LC in the cation-exchange mode. We used the cationic nature and inherent fluorescence of TBZ in the development of an analytical procedure for the determination of TBZ residues in whole, green bananas and ripe banana pulp.

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Experimental

Apparatus

(a) *Homogenizer.*—Food processor, Model 84186 (Hobart Corp., Troy, OH) and Waring commercial blender, Model 31BL41 (Waring Products Division, New Hartford, CT).

(b) *Variable-speed touch mixer.*—Vortex-Genie, Model K-550-G (manufactured for Fisher Scientific by Scientific Industries, Inc., Bohemia, NY).

(c) *Reciprocating shaker.*—Model 6000 (Eberbach Corp., Ann Arbor, MI).

(d) *Centrifuge.*—IEC Model HN-SII (International Equipment Co., Needham Heights, MA).

(e) *Vacuum manifold.*—United Chemical Technologies, Horsham, PA.

(f) *SPE column.*—BondElut PRS (propylsulfonic acid); 500 mg/2.8 mL (Varian Sample Preparation Products, Harbor City, CA).

(g) *LC column.*—PartiSphere SCX (benzenesulfonic acid); particle size = 5 μm; 12.5 cm × 4.6 mm (Whatman Inc., Clifton, NJ).

(h) *LC column temperature controller.*—Goldenfoil CH-1530 basic Model column heater (Systec, Inc., Minneapolis, MN).

(i) *LC system.*—Model 114M solvent delivery module/analytical (Beckman Instruments, Inc., Fullerton, CA);

WISP Model 710B autosampler (Waters Associates, Milford, MA); fluorescence monitor Model RF-551 (Shimadzu Corp., Japan); ChromJet integrator/SP4400 integrator (Thermo Separation Products, San Jose, CA).

(j) *LC conditions.*—Flow rate, 1.0 mL/min; injection volume, 20 µL; column temperature, 25.0°C; detector, 305 nm excitation, 380 nm emission, 1.5 s response, high sensitivity.

Reagents

(a) *TBZ reference standard.*—Purity, 99.8% (Merck & Co., Inc., Rahway, NJ).

(b) *Ethyl acetate, methanol (MeOH), acetonitrile (ACN), and water (H_2O).*—LC grade.

(c) *Phosphoric acid, H_3PO_4.*—85%, reagent grade.

(d) *KH_2PO_4, anhydrous Na_2SO_4.*—Analytical reagent grade.

(e) *SPE column conditioning solution.*—Transfer 1 mL H_3PO_4 (85%) to a 100 mL volumetric flask; add 20 mL water and dilute to 100 mL with methanol.

(f) *SPE column elution solution.*—Dissolve 14 g KH_2PO_4 in 700 mL water and dilute to 1 L with acetonitrile.

(g) *LC mobile phase.*—Dissolve 6.8 g KH_2PO_4 in 700 mL water and dilute to 1 L with acetonitrile. Adjust the pH of the solution to 3.8–4.0 with H_3PO_4.

TBZ Standard Solutions

(a) *TBZ stock solution (500 µg/mL).*—Dissolve 25 mg TBZ in 50 mL acetonitrile.

(b) *TBZ intermediate standard solution (5 µg/mL).*—Dilute 0.50 mL TBZ stock solution to 50 mL with acetonitrile.

(c) *TBZ working standards (2.5, 5.0, 7.5, and 10 ng/mL).*—Transfer 50, 100, 150, and 200 µL aliquots of TBZ intermediate standard solution (5 µg/mL) to individual 100 mL volumetric flasks and dilute each flask to the 100 mL mark with mobile phase.

Sample Preparation

(a) *Fresh, green bananas.*—Cut the green banana fingers (12–15 fingers per test sample) into 2 in. lengths and cut and/or blend in a Hobart food processor. Weigh the banana homogenate and transfer to a Waring-type blender jar. Add an equal weight of water and homogenize the mixture. Transfer 10.0 g banana homogenate (1 part banana to 1 part water) to a 50 mL centrifuge tube.

(b) *Ripe banana pulp.*—Place the ripe banana on a clean cutting surface, and with a clean, sharp knife, remove the crown end so that the banana can be peeled. Holding the opposite end of the whole banana, slowly strip approximately one-half of the peel from the pulp of the banana in such a manner that the pulp does not come in contact with the outer part of the peel or with the hands of the operator. While holding the end of the partially peeled banana, use a disposable plastic knife to remove a small portion of the pulp from the crown end of the banana. This is done to remove any of the pulp that may have been contaminated during the initial removal of the crown. Continue peeling the banana until ca 90% of the banana pulp is exposed. Break off the pulp from the peel into a suitable container. Collect 12–15 banana pulp fingers for each test sample, record the sample weight, and transfer to a Waring-type blender jar. Add an equal weight of water to the ripe banana pulp and homogenize the mixture. Transfer 20.0 g pulp homogenate (1 part pulp to 1 part water) to a 50 mL centrifuge tube.

Extraction and Cleanup

Add 25 mL ethyl acetate to the banana homogenate (whole banana and pulp), cap and shake on a reciprocating shaker for 10 min. Centrifuge at 2400–3200 × *g* (3500–4000 rpm) for 10 min. Transfer a 15–20 mL aliquot of the ethyl acetate extract to a clean 50 mL centrifuge tube. Add 2 g anhydrous Na_2SO_4 and manually shake for ca 5 s. Add more Na_2SO_4 if the ethyl acetate is not clear. Transfer 10.0 mL dried extract to a 2.8 mL PRS SPE column preconditioned with (*1*) 10 mL 1% H_3PO_4 in MeOH–H_2O (80 + 20), (*2*) 2 mL MeOH, and (*3*) 5 mL ethyl acetate. Successively wash the SPE column with (*1*) 5 mL ethyl acetate and (*2*) 3 mL MeOH. Discard the washes. Elute the TBZ from the SPE column with 9 mL 0.1M KH_2PO_4 in ACN–H_2O (30 + 70). Collect the eluate in a 10 mL volumetric flask and dilute to 10 mL with mobile phase.

Determination

Dilute appropriate volumes of the final sample extract with the mobile phase to give a TBZ concentration of ca 5–10 ng/mL. Determine the linear regression coefficients for the standard calibration curve from the plot of TBZ chromatographic peak responses (area or height) vs the corresponding concentration (ng/mL) of the TBZ working standards (2.5–10 ng/mL). Curves should be linear with a coefficient of determination (r^2) greater than 0.98. Calculate the amount of TBZ in the sample according to the following equation:

$$\text{TBZ in sample (ppm)} = \frac{C \times V \times 25}{W \times 1000}$$

where C = TBZ in LC solution (ng/mL), V = volume (mL) to which 1 mL final extract is diluted or the dilution factor (V is equal to 1 if no further dilution of the sample extract is made), and W = weight (g) of sample (W is equal to 5 for whole banana and 10 for banana pulp).

Fortifications

Untreated whole green bananas were of the Cavendish type grown at a commercial banana plantation. Banana pulp was obtained from untreated bananas after ripening in a commercial ripening house. Homogenates of the whole green banana and ripe banana pulp were fortified with TBZ at 0.05–10 ppm and 0.01–2 ppm, respectively. For each sample matrix, controls (0 ppm TBZ) and fortified samples were extracted and analyzed for TBZ. The percent recovery of TBZ from each matrix was determined by comparison of the amount of TBZ added to the amount of TBZ found.

Results and Discussion

The assay procedure described provides a simple, rapid, and sensitive method for monitoring TBZ residues in whole green bananas and ripe banana pulp. LC analysis of samples containing TBZ residues gave sharp TBZ peaks with an elution time of approximately 6 min. Figures 1 and 2 show typical chromatograms for TBZ in untreated whole green bananas and ripe banana pulp fortified with TBZ at 0.05 ppm. No chromatographic interferences from the control banana matrix were observed in the region of TBZ elution for either the untreated whole green banana or banana pulp. Standard calibration curves were linear ($r^2 > 0.98$) over a range of 0.05–0.2 ng TBZ injected on the column. The limit of quantitation, defined as the lowest TBZ fortification level for which recovery data were deemed acceptable, was 0.05 ppm for TBZ in whole green bananas and 0.01 ppm for TBZ in ripe banana pulp. The limit of quantitation could conceivably be much lower than those stated, because control green bananas and banana pulp gave apparent TBZ residues of <0.001 ppm. However, no recoveries of TBZ below the 0.05 ppm level in green bananas and the 0.01 ppm level in banana pulp were determined. Because the method uses fluorescence detection to quantitate TBZ residues, other pesticides or fungicides that are not inherently fluorescent are not expected to interfere with the analysis for TBZ.

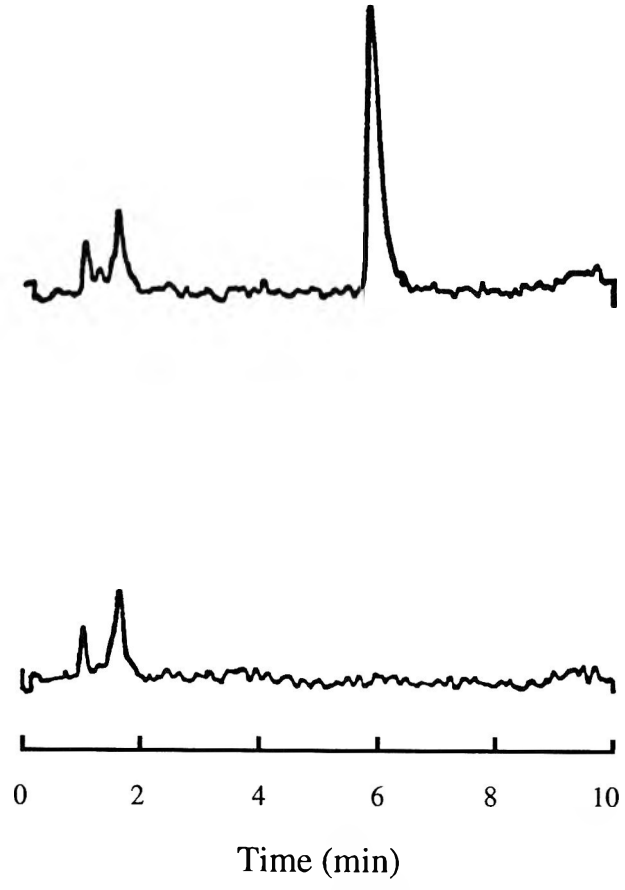

Figure 1. Typical chromatograms for whole green banana fortified with TBZ at 0.05 ppm (top) and control (0 ppm TBZ) whole green banana (bottom). The retention time for TBZ is 6.03 min. Approximately 0.1 ng of TBZ was injected on the column.

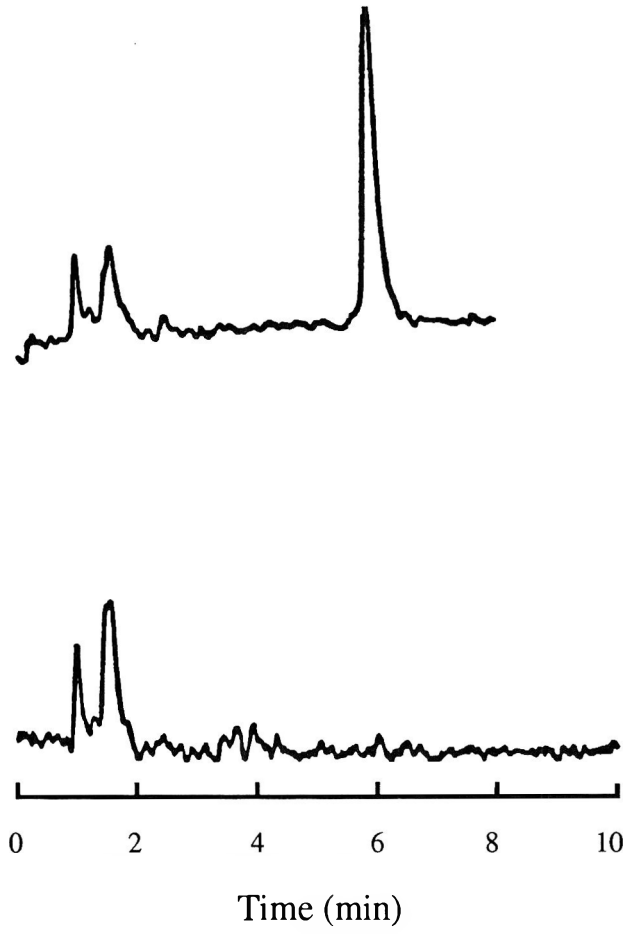

Figure 2. Typical chromatograms for banana pulp fortified with TBZ at 0.05 ppm (top) and control (0 ppm TBZ) banana pulp (bottom). The retention time for TBZ is 5.99 min. Approximately 0.1 ng of TBZ was injected on the column.

Table 1. Recovery of thiabendazole from fortified green bananas

Fortification level, ppm	Recovery, %	Mean, %	SD	CV (1σ), %
0.05	83.5	88.8	7.42	8.4
	94.0			
1	86.2	90.8	6.58	7.2
	95.5			
3	94.0	96.4	2.11	2.2
	97.5			
	97.8			
10	95.6	93.8	2.11	2.2
	91.5			
	94.4			

Table 2. Recovery of thiabendazole from fortified ripe banana pulp

Fortification level, ppm	Recovery, %	Mean, %	SD	CV (1σ), %
0.01	102	101	7.44	7.4
	93.2			
	108			
0.05	100	101	5.48	5.4
	107			
	96.2			
0.4	87.1	90.8	6.21	6.8
	87.4			
	98.0			
2	92.0	87.6	4.07	4.6
	86.7			
	84.0			

Recoveries of TBZ from untreated whole green bananas fortified with TBZ at 0.05–10 ppm and from banana pulp fortified with TBZ at 0.01–2 ppm are tabulated in Tables 1 and 2, respectively. Recoveries of TBZ from whole green bananas ranged from 83.5 to 97.8%, with an average recovery of 93% and a coefficient of variation (CV) (1σ) of 5.1%. Recoveries of TBZ from ripe banana pulp ranged from 84.0 to 108%, with an average recovery of 95% and a CV (1σ) of 8.5%.

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RESIDUES AND TRACE ELEMENTS

Analysis of Heavy Metals in *Aceto Balsamico Tradizionale di Modena* by Flame Atomic Absorption Spectroscopy

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A detailed investigation of *Aceto Balsamico Tradizionale di Modena* (ABTM) was carried out. The necessity of knowing the metal content of this original and widely used product is of great importance. Measurements were carried out on a large number of samples of different ages to check the dependence of metal content on product aging.

The heavy metal content of *Aceto Balsamico Tradizionale di Modena* (ABTM) was evaluated for a large number of samples characterized by different origin and age. ABTM is an Italian product with unique properties and exclusively manufactured in the district of Modena. The production of ABTM is extremely complex, because many factors are involved in the transformation of cooked musts into a highly aromatic but subtle condiment, whose scent and flavor are unique.

The last decades have witnessed an explosive increase in our knowledge of the elements essential for life and maintenance of plants, animals, and humans (1). The aims of the present work were to determine the amounts of chromium, nickel, copper, zinc, cadmium, and lead in ABTM and to correlate the sample heavy metal content with origin and age. The experimental results were compared with the limits imposed by the government to verify the hygienic and toxicological status of ABTM.

Generally, data about the metal content of foods and wines are largely reported in the specialized literature. Unfortunately, only few and incomplete data are listed for metals in musts and balsamic vinegars (2). All the metal cations investigated in the present study should be considered as micronutrient elements owing to their importance in many enzymatic complexes and biological systems. However, their monitoring is necessary because of their toxicity when present in high concentrations. Furthermore, some heavy metals are selectively concentrated in the human body in varying amounts. Studies during 1957–1980 by the late Klaus Schwarz (3) resulted in evidence supporting the essentiality of selenium, chromium, tin, vanadium, fluorine, silicon, nickel, lead, cadmium, arsenic, and, more recently, lithium.

Frequent tests of a large number of ABTM samples require a sensitive, precise, accurate, rapid, and cheap method. Quantitative determination of heavy metals in food and condiments is often accomplished by atomic absorption spectroscopy (AAS) (4, 5). Before measuring the quantity of metals in a particular sample, the ABTM must be digested to release the metal under investigation. This digestion process is the limiting factor for the time required by the entire procedure and also for the efficiency of recovering the actual amount of metal present in the sample (6). Metals can be released from the organic matrix by using heat and concentrated acid (wet ash method). Unfortunately, this technique has certain limitations, including excessive time, possible loss of metal by volatilization, excessive manipulation of sample, and incomplete digestion. However, as a first approach, we chose the wet ashing method as the only technique available for the treatment of a large number of samples.

We undertook a long-term study to check and monitor the chemical and physical parameters of ABTM related to the natural aging processes. We started with metal content determination to try to correlate the total amounts of the metals in the sample and the aging of the products.

Experimental

Apparatus

(a) *Glassware.*—Wash all glassware with nitric acid (HNO_3) (50%, v/v); rinse with cold tap water first and then with deionized water.

(b) *Atomic absorption spectrometer.*—Philips Pye Unicam Pu 9000 equipped with a single-slotted burner head and hollow cathode lamps. Wavelengths (nm) used were as follows: Cr, 357.9; Ni, 341.5; Cu, 324.8; Zn, 213.9; Cd, 228.8; and Pb, 283.3. Slit setting was at 0.5; air–acetylene with oxidizing (lean blue) flame was used for Pb, Cd, Cu, Zn, and Ni, and reducing flame (rich yellow) was used for Cr.

Reagents

All reagents were analytical reagent grade. Deionized water, obtained from a Milli-Q plus (Millipore) apparatus, gave

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specific conductivity values of ≤55 nS cm^{-1} and was used throughout.

(a) *Nitric acid.*—70% (v/v).

(b) *Atomic absorption reference solutions.*—1000 ppm (Carlo Erba Milan).

Preparation of Standards

(a) *Atomic absorption working standards.*—Mixed working standards of Pb, Cd, Ni, Cu, Zn, and Cr were prepared by diluting 1000 ppm stock reference solutions to produce concentrations ranging from 0.05 to 8.0 ppm.

(b) *Sample spiking standard solutions.*—Spiking standards of Cr (10 ppm), Ni (5 ppm), Cu (5 ppm), Zn (50 ppm), Cd (2.5 ppm), and Pb (5 ppm) were prepared by diluting 1000 ppm stock reference solutions.

Analytical Procedure

(a) *Sampling.*—The 99 ABTM specimens were sampled from 10 batches of barrels. From each batch of barrels of different size, different wood, and different product aging, an aliquot of about 100 mL of ABTM was taken.

(b) *Method procedure.*—Because of the density of the product, each determination was carried out by weight to avoid any volume correction of the final data. Furthermore, at the time of analysis, each sample was stirred at room temperature to dissolve and homogenize the solid particles formed during storage.

Each sample was analyzed twice for heavy metals. A control blank containing reagents only was also analyzed.

(c) *Wet ashing.*—A sample portion was weighed into a 250 mL Pyrex beaker, and concentrated nitric acid (30 mL) was carefully added. Blank samples containing only nitric acid were run concurrently throughout the entire procedure. Following overnight predigestion at room temperature, samples were placed on a sand bath at 60°C and digested until a dark orange color appeared. The volume was reduced to near dryness by gentle boiling. Charring of samples was avoided to minimize the possibility of analyte loss. An additional 10 mL of concentrated nitric acid was added to samples and blanks, and digestion was continued until approximately 5 mL of solution remained. The insides of beakers were rinsed with 10–15 mL of deionized water and the samples were heated for an additional 10 min. Samples were then removed from heat, cooled to room temperature, brought to volume in a 25 mL volumetric flask with deionized water, and then filtered through filter paper (Schleicher & Schuell 588 prepleated, Keene, NH).

(d) *Determination.*—Determination of heavy metals in the prepared samples was performed with flame AAS. Samples analyzed for all the metals were run without further dilution. The dilution factor was 2.5 (10 g of sample in a total volume of 25 mL). Standards and samples were run under the same instrumental conditions as previously described. Standard curves for each element under investigation were run at the beginning and end of each session. The spectrophotometer made corrections for background interferences. Samples, standards, and blanks were aspirated until maximum peak heights were obtained and a flat reading was produced on the display. Water was aspirated after each sample, blank, or standard until the reading returned to baseline.

(e) *Calculations.*—Regression curves were fitted to the plots of absorbance reading versus the concentration (ppm) of each analyte. Concentrations of metals in each sample were calculated from the corresponding regression equations and dilution factors (weight to weight).

(f) *Recoveries from spiking samples.*—To check the accuracy of the wet ashing method, recoveries of the investigated metals from the ABTM matrix were carried out. The determination of metals was done on an ABTM matrix obtained by mixing several vinegar samples of different aging. Four aliquots of roughly 10 g each were weighed into 250 mL Pyrex beakers, and 3 of these were spiked with 1, 2, and 3 mL of the spiking standard solutions. These samples were treated by the procedures cited above. To improve the precision of the recovery data, all the samples were prepared in duplicate. The resulting solutions were analyzed by flame AAS. From the linear regression between the concentration data obtained from the metal readings against the additions of each element, referred to the unit mass of the ABTM (mg/kg), the slope of the straight line represents the recovery of the metal averaged over 4 determinations.

Results and Discussion

The present study is the first detailed investigation of ABTM, a typical product with a great tradition in the region of Modena. In this work we considered 6 metals. Of these, chromium, nickel, copper, and zinc are normally classified as essential trace elements for the human body because their presence is fundamental for the biochemistry of metalloproteins and metalloenzymes. The other 2 metals (cadmium and lead) are normally considered harmful to human health (7). Although these metals are required only in ultratrace quantities, their deficiency can lead to growth suppression, anemia, reduced reproduction, etc.

Only ABTM older than 12 years can be put on the market by the Italian Union of the Producers of ABTM; therefore, metal enrichment during the aging process needs to be determined.

Table 1 summarizes the results obtained from 99 ABTM samples relative to the 6 investigated metals. For clarity, the samples were divided in 4 groups depending on the age of the product, starting from the cooked must (raw material) to the oldest ABTM.

The trend of metal content is reported in Table 1. Chromium concentration, after an initial increase to about 1 ppm in the initial mellowing period, remained almost constant during the aging period. On the contrary, nickel concentration did not increase with time.

Copper rapidly decreased in the initial mellowing period and remained quite constant up to about 1 ppm for the marketable product. Zinc increased progressively during aging inside the barrels. Cadmium and lead showed similar trends during the mellowing process. Their concentrations remained quite

Table 1. Metals found in ABTM samples at various stages of aging

Sample or aging stage	n	Amount of metal, mg/kg																					
		Cr				Ni				Cu				Zn				Cd				Pb			
		Min.	Av.	Max.	SD	Min.	Av.	Max.	SD	Min.	Av.	Max.	SD	Min.	Av.	Max.	SD	Min.	Av.	Max.	SD	Min.	Av.	Max.	SD
Cooked must[a]	5	Tr[b]	Tr	Tr	Tr	Tr	0.60	1.30	0.43	Tr	6.31	9.19	3.69	1.87	4.32	7.85	2.40	Tr	Tr	Tr	Tr	Tr	0.25	0.61	0.23
1–6 years[c]	20	Tr	1.01	3.40	0.89	Tr	0.44	1.40	0.44	Tr	1.11	4.80	1.34	1.80	8.55	25.00	6.13	Tr	0.23	0.80	0.19	Tr	0.50	3.80	1.01
6–12 years[d]	28	Tr	0.69	5.60	1.10	Tr	0.45	1.40	0.53	Tr	1.41	3.40	1.15	2.80	12.74	31.80	6.59	Tr	0.13	0.40	0.14	Tr	0.28	2.00	0.50
>12 years[e]	46	Tr	0.97	7.03	1.11	Tr	0.60	2.80	0.65	Tr	1.36	4.60	1.28	5.60	18.94	48.40	11.40	Tr	0.22	1.90	0.34	Tr	0.53	3.40	0.78

[a] n = 5
[b] Tr = Trace
[c] n = 20
[d] n = 28
[e] n = 46

Table 2. Metals found in must sediments[a]

Sample[b]	Amount of metal, mg/kg					
	Cr	Ni	Cu	Zn	Cd	Pb
A	0.71	0.84	156.9	3.31	0.33	2.32
B	0.32	0.70	15.66	6.23	0.09	4.81
C	Trace	0.52	12.13	3.53	0.06	0.47

[a] Data are averaged values.
[b] A, Sediments obtained from the must just squeezed and not cooked; B, rough sediments obtained from the cooked must; C, thin sediments obtained from the stored must.

constant up to about 0.2 ppm and about 0.5 ppm for cadmium and lead, respectively.

ABTM is one of the Italian products with the D.O.C. trademark (Checked Denomination and Origin), which establishes the kind of raw materials (cooked musts) and all the aging procedures required to obtain the final product. Even if ABTM is made according to the D.O.C. trademark, no information exists on elemental, in particular heavy metal, composition. The only data available that can be compared with the metal concentrations in ABTM (Table 1) are the limits of amounts of metals contained in wine (8) and in vinegars (9). Italian law establishes the limits for copper, zinc, and lead at 1, 5, and 0.3 ppm, respectively. These values may be compared with the data in Table 1 referring to the oldest samples, which are the marketable product.

The values obtained for zinc and lead in ABTM are generally higher than the limits established by law, whereas the values for copper are quite close to the imposed limits. However, this comparison is not valid, because the two products being compared, ABTM and wine, have different chemical properties and different alimentary uses. Wine is usually considered a moderately alcoholic drink, which may be consumed in considerable quantities. On the other hand, ABTM is considered a highly aromatic condiment whose scent and flavor are unique and, for these reasons, is always used in very small quantities. Moreover, the procedures followed in the production of vinegar and ABTM are completely different. In fact, in the first case the raw material is wine, which undergoes acetic acid fermentation. The product is not subject to a concentration phenome-

Table 3. Recommended safe and adequate dietary intakes for adults

Element	Intake, mg/day	Ref.
Chromium	0.02–0.5	11
Nickel[a]	0.1–0.8	12
Copper	2.0–3.0	13
Zinc	15	13, 14
Cadmium[b]	0.057–0.071	15
Lead[b]	0.430	16

[a] Average intake.
[b] Maximum values.

Table 4. Average recoveries from spiked samples in ABTM matrix

	Cr, mg/kg		Ni, mg/kg		Cu, mg/kg		Zn, mg/kg		Cd, mg/kg		Pb, mg/kg	
Parameter	Added	Found	Added	Found	Added	Found	Added	Found	Added	Found	Added	Found
	0	0.90	0	1.04	0	0.96	0	11.3	0	0.28	0	0.62
	0.51	1.41	0.51	1.48	0.51	1.49	5.13	16.9	0.26	0.55	0.51	1.13
	1.01	2.01	1.01	1.98	1.01	1.95	10.1	22.1	0.51	0.82	1.01	1.72
	1.48	2.50	1.48	2.44	1.48	2.44	14.8	27.3	0.74	1.07	1.48	2.13
Recovery	1.09		0.951		0.992		1.08		1.07		1.04	
Corr. coeff.	0.998		0.998		0.999		0.999		0.999		0.997	

non. As a consequence, it is reasonable to think that the metal concentration remains unchanged. ABTM production starts from selected grape must; the filtered must is concentrated by direct flame cooking in stainless steel containers, and then the cooled, cooked must is added to the largest barrel for aging.

The mechanical processes and the sedimentation periods of the must result in a decrease in the concentration of metals in the liquid product, as can be deduced from Table 2, where the metal content of the sediments obtained from the different working phases of the must are reported. Concentrations of metals in must are greatly reduced during the first and second working phases, which are the filtration of the squeezed must and the first sedimentation after cooking, respectively. Hence, the major differences between wine vinegar and ABTM are the starting raw material and the mellowing process, which are certainly responsible for the unique aroma and flavor of ABTM. Moreover, the mellowing process involves the concentration of the product and consequently an increase of the concentration of inorganic components. ABTM undergoes a volume reduction of about 4–5 times.

This concentration effect is evident from the data in Table 1, which shows an increase in the concentration of chromium and zinc on passing from the cooked must to the marketable product. The concentrations of other metals remained constant in the same period. For these elements, we may think of a different mechanism that leaves constant the metal concentration in the liquid phase and increases the metal concentration in the so-called "mother" of ABTM. In fact, the ABTM mother is the biochemical agent for all the fermentative processes, where the enzymes probably link metals such as nickel and copper to form metalloenzymes having catalytic function (10). The same process is valid for cadmium and lead.

For these reasons the metal content of ABTM may be considered acceptable, because of the fact that daily intake of ABTM is very limited. For an average daily consumption of 5 mL of ABTM, the absolute quantity of metals ingested is always considerably below the recommended safe and adequate dietary intake values as reported in Table 3 (11).

To verify the accuracy of the wet ashing technique, spike recoveries were determined for the 6 metals under investigation. Because of the absence of a reference matrix similar to that used in this work, we chose to mineralize different aliquots of ABTM containing known additions of the different metals. In Table 4, the average recoveries are reported for the 6 investigated metals. The results confirm the absence of particular matrix effect and any loss of material, with recoveries quite close to 100%.

Conclusion

The results of this study show that the mellowing of ABTM, which confers and increases its scent and flavor, increases the metal content of the marketable product. Nevertheless, comparison of the absolute amounts of metal consumed and the recommended dietary intakes shows that ABTM is acceptable, even though the metal concentrations are over the legal limits for wine and wine vinegar, because the daily assumption of the product is very small.

Acknowledgments

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RESIDUES AND TRACE ELEMENTS

Rapid Abrasion Test To Indicate Lead on the Surface of Ceramicware

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A rapid abrasion test (RAT) was developed for screening ceramicware for Pb. A Pb-sensitive chromogen (rhodizonic acid) reacts to form a persistent colored complex, indicating the presence of Pb. RAT takes 2–5 min to complete; a positive test is easily discernible. RAT, which provides only qualitative information, is useful for screening large numbers of ceramicware items for the presence of Pb at ≥0.05 μg/mL.

Since the late 18th century, certain ceramic glazes have been recognized as potential sources of dangerous Pb levels when used with acidic or alkaline foods (1). The U.S. Food and Drug Administration (FDA) routinely monitors both domestic and imported ceramicware (2, 3). The method of AOAC INTERNATIONAL (4) for Pb and the equivalent method of the American Society for Testing and Materials (5) are used for testing ceramicware for leachable (extractable) Pb. Both methods require leaching of ware for 24 h with 4% acetic acid at room temperature. The leachate is analyzed for Pb by flame atomic absorption spectroscopy.

The quick color test (QCT), developed (6) and patented (U.S. Patent 4 873 197 and 5 010 020) by FDA, has been used successfully by FDA field and laboratory personnel as a screening test for foodware to determine the need for analysis by the AOAC Official Method. FDA has recently lowered the action levels for maximum Pb release from ceramicware (7, 8) (Table 1). Ware found to contain Pb at or above these levels is considered for regulatory action. The sensitivity of the QCT was determined to be insufficient for screening some types of ware at the new action levels (9) and prompted development of a more sensitive test.

A rapid abrasion test (RAT) was developed for screening ceramicware for Pb. RAT uses the Pb-indicating chromogen of the QCT (6) to qualitatively measure Pb in glaze abraded from the ware surface. The QCT and the AOAC Official Method screen ware for leachable Pb only. RAT is a more stringent test for Pb because abrading the glaze increases the surface area leached.

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METHOD

Principle

The food contact surface of ceramicware is abraded with sandpaper. Citric acid solution is used to extract Pb from the abraded glaze. A Pb-sensitive chromogen (rhodizonic acid) reacts to form a persistent pink complex, which indicates the presence of Pb (10).

Reagents and Materials

All reagents are ACS reagent grade unless otherwise indicated. Water for reagent preparation should be either distilled or deionized. Commercially available kits (FRANDON Lead Alert Kit, Frandon Enterprises, Inc., Seattle, WA, or equivalent) also may be used.

(a) *Citric acid solution, 1.3%.*—Dissolve 1.3 g citric acid in 100 mL water. Mix well to dissolve. This solution is stable

Table 1. FDA action levels for Pb released from ceramicware

Category	Old action level, μg Pb/mL (7)	Current action level, μg Pb/mL (8)
Flatware	7.0	3.0
Small hollowware[a]	5.0	2.0[b]
Cups and mugs[a]	—	0.5
Large hollowware[c]	2.5	1.0[d]
Pitchers[c]	—	0.5

[a] Volume, <1100 mL.
[b] Excluding cups and mugs.
[c] Volume, ≥1100 mL.
[d] Excluding pitchers.

for several months. However, if mold growth appears, discard solution. Refrigeration will extend reagent life. To ship kits and undiluted reagents, obtain dried and preweighed citric acid in polyethylene bottle of known volume (Nalgene, Nalge Co., Rochester, NY, or equivalent).

(**b**) *Detergent wash.*—Use detergent formulated for washing dishes by hand to wash ware for laboratory testing. Mix with lukewarm water according to product instructions. For field testing, merely wipe ware free of particulate matter.

(**c**) *Chromogen solution, 0.2%.*—Preweigh ca 0.05 g rhodizonic acid dipotassium salt (Cat. No. 115 4608, Eastman Kodak Co., Rochester, NY, or equivalent) in 25 mL, disposable polyethylene scintillation vial with screw cap (Cat. No. 16022-274, VWR Scientific, S. Plainfield, NJ, or equivalent). Add water to dissolve solid and dilute to neck of vial just before use; mix thoroughly. Depending on the source of rhodizonic acid, undissolved residue may remain. Protect solution from light and excessive exposure to air. Prepare fresh solution daily or more frequently if solution darkens. Apply drop of solution to untreated filter paper. If spot is not bright yellow, reagent should be discarded.

(**d**) *Sandpaper squares.*—Cut sandpaper, 220-grit silicon carbide (3M, or equivalent) into ca 2.5 × 2.5 cm (1 × 1 in.) squares.

(**e**) *Filter paper squares.*—Cut 46 × 57 cm filter paper (Whatman No. 1 chromatographic paper, Cat. No. 28451-006, VWR Scientific, or equivalent) into ca 2.5 × 2.5 cm (1 × 1 in.) squares.

(**f**) *Disposable pipets.*—3.5 mL polyethylene transfer pipets (Cat. No. 14670-205, VWR Scientific, or equivalent). Use separate pipets to apply chromogen and citric acid solutions. Citric acid rapidly degrades rhodizonic acid when Pb is not present.

Preliminary Analysis: Radioisotope-Induced X-ray Emission Spectrometer

^{109}Cd excitation source (New England Nuclear Division of DuPont, Boston, MA); planar Ge(Li) photon detector (EG&G Ortec, Oak Ridge, TN); multichannel analyzer (Model 6620, Nuclear Data, Inc., Schaumburg, IL). Use collimation system with resolution of ca 4 mm diameter to test small surface areas. Accumulation times of X-ray spectra ranged from 1 to 30 min. Surface Pb was measured on selected cups by radioisotope-induced X-ray emission (RIXE) spectrometry before other tests were performed to identify cups that contained Pb as main component of glaze.

Testing Procedure

(**a**) *Ware preparation.*—In the laboratory, clean each item with detergent solution and rinse first with tap water and then with distilled or deionized water, and air-dry. In the field (warehouse, etc.), merely wipe ware surface with clean cloth to remove particulate matter.

(**b**) *Abrasion of food contact surface.*—Abrade small area (ca 1–2 $in.^2$) of food contact surface with sandpaper square by pressing firmly with thumb and rubbing until sandpaper is covered with abraded surface dust (ca 0.5–3 min, depending on surface hardness). Application of RAT to decorated ware requires that each glaze color, decal decoration, and background of item be tested until either a positive result is found or all areas have been tested. Undecorated ware is tested in only one area. (*Note*: If many items are to be tested at one time, cover thumb with masking tape or glove to minimize irritation.)

(**c**) *Placement of filter paper.*—Place 1 filter paper slip on abraded area of ceramicware and another slip on sandpaper. Using pipet, wet slips with several drops of citric acid. Slips must be in direct contact with surface (i.e., no ridges or bubbles) for Pb to diffuse into citric acid.

(**d**) *Color development.*—Add several drops of chromogen solution to paper slips. A positive test is indicated by a pink spot that forms immediately after chromogen application on either slip. Color may persist for hours. A negative test is indicated by an initially yellow spot that fades fairly rapidly (ca 1–2 min) on both slips. If underlying glaze colors interfere with viewing developed color, remove wet paper from ware and place on clean white surface.

Results and Discussion

Undecorated ware was chosen for this study, because it offered the most thorough comparison of RAT and the AOAC Official Method. Undecorated ware has leach characteristics that are generally uniform across the surface, whereas those of decorated ware vary with the surface areas of the decorations. Pb that is concentrated on a small decoration is detected more easily at low levels with RAT. The presence of decorations makes no difference in the AOAC 24-h extraction protocol (4) because the entire surface is leached. The exact sensitivity of RAT for Pb in a piece of decorated ware depends on the surface area of the Pb-releasing decoration(s) and whether Pb is released by the undecorated surface. Every decorated ware tested was positive in RAT; the leach concentrations obtained with the AOAC Official Method ranged from <0.2 (i.e., detection limit) to >10 000 μg Pb/mL.

RAT was used to test 65 cups representing 50 varieties of undecorated ceramicware and 18 pieces of decorated ceramicware, earthenware, and glassware (plates, bowls, and spoons). RAT also was used to test cleaned quartz beakers to

Table 2. Results of cup and mug testing by RAT and the AOAC leaching procedure

Cup	RAT result	Leach solution, µg Pb/mL	Cup	RAT result	Leach solution, µg Pb/mL
113-1	POS	534	159-1	POS	0.019
162-1	POS	2.5	175-1	POS	0.016
112-1	POS	1.4	146-1	POS	0.008
84-6	POS	1.3	155-1	NEG	0.006
139-5	POS	1.0	145-1	NEG	0.004
84-4	POS	1.0	181-1	NEG	0.004
139-6	POS	0.84	151-1	NEG	0.003
139-2	POS	0.66	163-1	NEG	0.003
139-4	POS	0.63	178-1	NEG	0.003
139-3	POS	0.62	184-1	NEG	0.003
139-1	POS	0.62	148-1	NEG	0.002
84-3	POS	0.53	164-1	NEG	0.002
84-2	POS	0.51	173-1	NEG	0.002
84-5	POS	0.49	183-1	NEG	0.002
84-1	POS	0.47	169-1	NEG	0.001
149-1	POS	0.47	141-1	NEG	<0.001
147-2	POS	0.23	143-1	NEG	<0.001
147-3	POS	0.18	150-1	NEG	<0.001
147-4	POS	0.18	152-1	POS	<0.001
176-1	POS	0.14	153-1	NEG	<0.001
147-1	POS	0.12	154-1	NEG	<0.001
142-1	NEG	0.12	158-1	NEG	<0.001
147-5	POS	0.11	160-1	NEG	<0.001
144-1	POS	0.070	161-1	NEG	<0.001
147-6	POS	0.069	165-1	NEG	<0.001
174-1	POS	0.064	166-1	NEG	<0.001
157-1	POS	0.052	167-1	NEG	<0.001
140-1	POS	0.047	168-1	NEG	<0.001
177-1	POS	0.047	170-1	NEG	<0.001
179-1	NEG	0.025	171-1	NEG	<0.001
156-1	POS	0.022	172-1	NEG	<0.001
182-1	NEG	0.022	180-1	NEG	<0.001
185-1	NEG	0.020			

preclude contamination from materials and reagents. Before being tested by RAT, each ceramicware was tested for leachable Pb by the AOAC 24-h extraction protocol (4), and the leach solutions were analyzed for Pb by graphite furnace atomic absorption spectrometry (GFAAS) (11).

Surface Pb was measured on selected cups by RIXE spectrometry to identify those that contained Pb as a glaze component. Results for RAT were in excellent agreement with those obtained by RIXE spectrometry for Pb at ≥0.020 µg/mL. At Pb levels of <0.020 µg/mL, results for RAT differed from those obtained by RIXE spectrometry for ca 15% of the cups tested. These findings may have been caused by differences in sampling depth, which would be more critical at lower levels. Cup 142-1 exhibited glaze defects after leaching, which may have allowed Pb to leach from the cup's clay. This possibility may explain why RAT and RIXE spectrometry detected no surface Pb even though leachable Pb was found at 0.12 µg/mL.

Table 2 lists the results obtained for undecorated ware by RAT and the AOAC Official Method. Concentrations of Pb found in the leach solutions are listed in descending order. Figure 1 displays the sensitivity of RAT as a function of RAT reliability and Pb concentration in the leach solution as determined by the AOAC method. The reliability of reporting a positive RAT result is defined empirically as follows:

$$\text{Reliability, \%} = (\text{No. of positive RAT results at or above a specific concentration/No. of cups tested at or above this concentration}) \times 100$$

The sensitivity of RAT was defined arbitrarily as the lowest Pb concentration at or above which RAT was at least 95% reliable. The sensitivity of RAT was determined to be 0.047 µg Pb/mL.

Extrapolation of the RAT sensitivity to other sizes and shapes of ware requires correction for differences in the surface area/volume ratio (*SA/V*). Ranges of *SA/V* have been estimated by FDA for different types of ware (12). RAT sensitivities were calculated for different types of ware by using the RAT sensi-

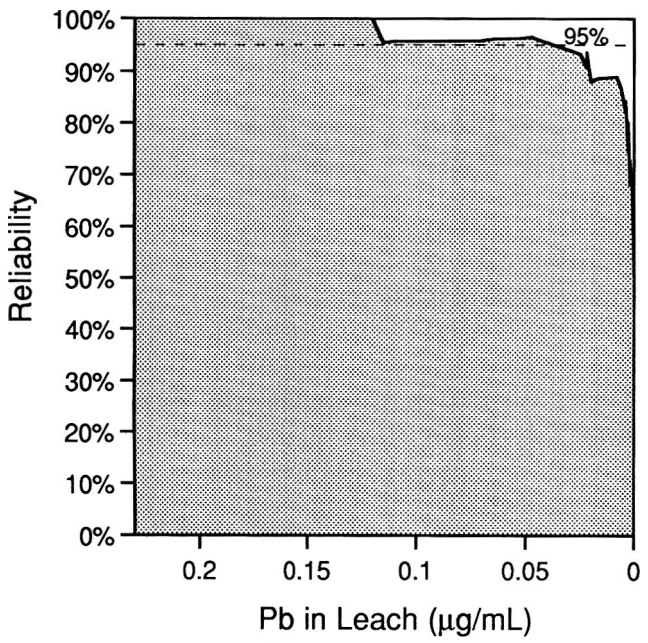

Figure 1. Sensitivity of the rapid abrasion test.

tivity rounded to 0.05 µg Pb/mL and the *SA/V* of cup 140-1, which leached at the RAT sensitivity level, according to the following equation:

$$\text{RAT sensitivity, } \frac{\mu g\ Pb}{mL} = \frac{0.05\ \mu g\ Pb}{mL} \times \frac{(SA/V)}{0.65\ cm^{-1}}$$

The results of the calculations are given in Table 3 and can be used as a guide when the *SA/V* is not known. If the *SA/V* is known for a particular undecorated ceramicware item, the RAT sensitivity can be estimated from the equation.

A test trial was conducted by 2 independent laboratories (Blaine Shishido, State of Hawaii, Department of Health, and Richard Jacobs, San Francisco District Office, FDA) to evaluate the performance of RAT in the field. One laboratory used GFAAS for Pb determination. A total of 48 pieces of ceramicware were tested by AOAC method **973.32** (4). Twenty pieces gave leach solution concentrations of <0.02 µg Pb/mL and tested negative by RAT. The remaining 28 pieces gave leach solution concentrations of ≥0.02 µg Pb/mL and tested positive by RAT. These results confirmed the sensitivity of RAT for laboratory and field use.

Table 3. RAT sensitivity for undecorated ceramicware[a]

Type of ware	Range of surface area/volume ratio, cm^{-1} (12)	Range of sensitivity, µg Pb/mL
Flatware	0.35–1.8	0.03–0.1
Small hollowware[b]	0.44–1.3	0.03–0.1
Cups and mugs[b]	0.37–1.0	0.03–0.07
Large hollowware[c]	0.37–0.55	0.03–0.04
Pitchers[c]	0.44–1.3	0.03–0.1

[a] Based on 24 h, 4% acetic acid leaching.
[b] Volume, <1100 mL.
[c] Volume, ≥1100 mL.

RAT is sensitive and specific for Pb. Potential chromogen interferences from other elements were discussed elsewhere (6). Broken ware may be tested by RAT, provided that the ware surface has not been compromised. Because RAT alters the surface of ware, the same piece of ware tested with RAT should not be tested by the leach protocol.

RAT is not sensitive to Cd, another element of regulatory concern (13). Ceramicware that bears decoration (e.g., pigmented glazes and glaze decals) in shades of yellow, orange, or red may contain Cd and should be tested for that element by an appropriate procedure.

RAT can indicate the presence of surface Pb at ≥0.05 µg Pb/mL on ceramicware with 95% reliability. RAT is an inexpensive and simple qualitative test for Pb that can be conducted with a portable kit. The time necessary to screen ceramicware by using RAT (<5 min) is much less than that required for the QCT (≤60 min) (9). The use of RAT can contribute significantly to the efficiency of locating potentially violative ceramicware and of eliminating from further testing ware that does not contain surface Pb.

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RESIDUES AND ELEMENTAL ANALYSIS

Determination of Zinc in Serum, Blood, and Ultrafiltrate Fluid from Patients on Hemofiltration by Graphite Furnace/Atomic Absorption Spectroscopy or Flow Injection Analysis/Atomic Absorption Spectroscopy

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Two methods were optimized for the determination of zinc in samples of blood, serum, and ultrafiltrate fluid from patients with chronic renal impairment undergoing hemofiltration. In the first procedure, after acid digestion of the samples, Zn in blood and serum is determined by a system coupled to flow injection analysis and atomic absorption spectroscopy. The method is rapid, automated, simple, needs small amounts of sample, and has acceptable analytical characteristics. The analytical characteristics obtained were as follows: determination range of method, 0.05–2.0 ppm of Zn; precision as coefficient of variation (CV), 5.3%; recovery, 95–105%; and detection limit (DL), 0.02 ppm. The second method is optimized for ultrafiltrate fluid because the sensitivity of the first procedure is not suitable for the levels of Zn (ppb or ng/mL) in these samples. The technique chosen was atomic absorption spectroscopy with electrothermal atomization in a graphite furnace. The analytical characteristics obtained were as follows: determination range of method, 0.3–2.0 ppb Zn; CV, 5.7%; recovery, 93–107%; and DL, 0.12 ppb. The methods were used to determine zinc in samples of blood, serum, and ultrafiltrate fluid from 5 patients with chronic renal impairment undergoing hemofiltration to discover whether there were significant differences in the zinc contents of blood, serum, and ultrafiltrate fluid after the hemofiltration process. An analysis of variance of the experimental data obtained from a randomly selected group of 5 patients showed that zinc concentrations in the ultrafiltrate fluid, venous blood, and venous serum do not vary during hemofiltration ($p < 0.05$), whereas in arterial blood and serum, the time factor has a significant effect.

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Treatment of patients with chronic renal impairment (CRI) by hemodialysis has permitted physicians to greatly prolong their survival and their quality of life. In recent years, several publications have drawn attention to the appearance of deficits or overloads of certain trace elements, such as Zn (1–2), in such patients. A deficiency of Zn in plasma may lead to abnormalities in bone metabolism, skin lesions,

testicular atrophy, impotence, deficient healing of wounds, and alternations in taste and smell.

The most recent analytical procedures for Zn determination in blood and serum combine in the continuous mode flow injection analysis (FIA) with microwave sample dissolution and atomic absorption spectroscopy (AAS). Although these continuous systems have several advantages, their cost is somewhat prohibitive for the usual laboratory apparatus setup (3).

For the determination of Zn by AAS with electrothermal atomization in a graphite furnace (GF/AAS), several reports have addressed optimization of analytical characteristics and improvement of accessories, such as the peak-shape monitoring system that avoids losses of volatile elements during the ashing stage (4). Our method has the novelty of determining the element within the matrix of the ultrafiltrate fluid from patients with chronic renal failure on hemofiltration; the effect of the corresponding salt matrix is avoided. In fact, another group of methods couples FIA with inductively coupled plasma/atomic emission spectrometry (5).

In the present work, we optimized a new procedure for the determination of Zn in samples of serum and blood by coupling AAS with FIA, which has advantages when sample amounts are low. The determinations were carried out after previous acid digestion of samples, followed by dilution in ultrapure water to minimize matrix effects. A second procedure was optimized for the determination of Zn in samples of ultrafiltrate fluid by GF/AAS, because the analytical signal from ultrafiltrate fluid was below the detection limit of FIA/AAS. Determination is carried out after diluting the sample in ultrapure water to minimize the effects of the saline matrix.

Experimental

Apparatus and Reagents

(a) *Sample digester.*—A digestion battery for Kjeldahl flasks (Selecta) was used for acid digestion of blood and serum samples.

(b) *Atomic absorption spectrophotometer.*—Varian AA-1475 with a Cathodeon zinc hollow cathode lamp.

(c) *Flow system.*—Coupled to the AAS detector, consisting of a Gilson Minipuls peristaltic pump (Model 2HP4) with polyvinyl chloride tubes (1.0 mm id), an injection valve (Model 5020, Rheodyne) fitted with loops of Teflon tubes (0.5 mm id), and "T" connectors and junctions.

(d) *Atomic absorption spectrophotometer with electrothermal atomization.*—A Varian GTA 95 equipped with an automatic sampler and pyrolytic graphite tubes.

(e) *Water purification system.*—Mod Elgastat UHQ for obtaining ultrapure water.

(f) *Cleaning of glassware.*—To eliminate the assumed contamination of Zn at such low levels, all materials used were previously washed and rinsed as follows:

(*1*) *For blood and serum.*—The glassware was washed in soap and rinsed, soaked in analytical reagent (A.R.) HNO_3 (1:20) for at least 8 h, and then rinsed 3 times with double-distilled water and then with ultrapure water.

(*2*) *For ultrafiltrate.*—The glassware was washed with soap and rinsed, soaked in A.R. HNO_3 (1:20) for at least 24 h, and then carefully rinsed 5 times with double-distilled water and then with ultrapure water. Ultrapure water also was used for preparing and diluting standards.

(g) *Standard solution of Zn^{2+} (1000 ppm, w/v).*—Prepared from zinc (II) chloride (Merck A.R.) by dilution in acid medium HNO_3 (A.R., 0.1%).

(h) *Nitric acid, 65%.*—Carlo Erba RPE.

(i) *Sulfuric acid, 96%.*—Carlo Erba RPE.

(j) *Hydrogen peroxide, 30%.*—Carlo Erba RPE.

(k) *Perchloric acid, 65%.*—Carlo Erba RPE.

Sample Collection

Blood samples (20 mL) were taken by venipuncture, using disposable polypropylene syringes and stainless steel needles. Half of each blood sample was transferred into a zinc-free 10 mL polystyrene tube containing lithium heparin. The remaining 10 mL of blood was placed in a plain polystyrene tube, and the serum was separated within 30 min of collection. Serum and blood were stored at 4°C until required for analysis.

Sample Treatment

Blood.—A sample of exactly 5 g blood was placed in a 125 mL Erlenmeyer flask, and 4 mL 65% HNO_3 and 1 mL 96% H_2SO_4 were added. The Erlenmeyer flask was placed in a Kjeldahl sample digester and heated gently until the froth disappears. The temperature of the Kjeldahl digester was raised to 130–150°C until the solution boiled and became dark (ca 1 h). After the flask cools to room temperature, 2 mL 65% HNO_3 plus 1 mL 65% $HClO_4$ were added, and heating was resumed until white fumes began to appear. The mixture was then cooled to room temperature, 1 mL 30% H_2O_2 was added dropwise, and the mixture was heated again but not above 180°C. Treatment with H_2O_2 completed the oxidation and removed excess HNO_3 in the sample solution that otherwise might interfere with the measurement (6–8). The residue of ca 2 mL was transferred quantitatively to a 10 mL volumetric flask; the sides of the flask were washed with ultrapure water, and the volume was brought up to 10.0 mL. After acid digestion, the sample was diluted 1:5 ultrapure water.

Serum.—The procedure was the same as for blood samples, except that H_2O_2 was not used and the amounts of acids were 3 mL 65% HNO_3 and 1 mL 96% H_2SO_4 in the first addition and 1 mL 65% $HClO_4$ in the second addition. After acid digestion, the samples were not diluted further.

Ultrafiltrate.—A 1:19 dilution was made first in ultrapure water. The sample was diluted again at 1:5 in ultrapure water by using the autosampler of the graphite furnace. The resulting dilution was 1:95.

Method I.—Determination of Zn in Blood and Serum by FIA/AAS

The design and components of the FIA/AAS coupled system are shown in Figure 1. The setup consists of 2 Teflon capillary tubes through which the samples and carrier (ultrapure water) are channeled with the aid of a peristaltic pump. Both

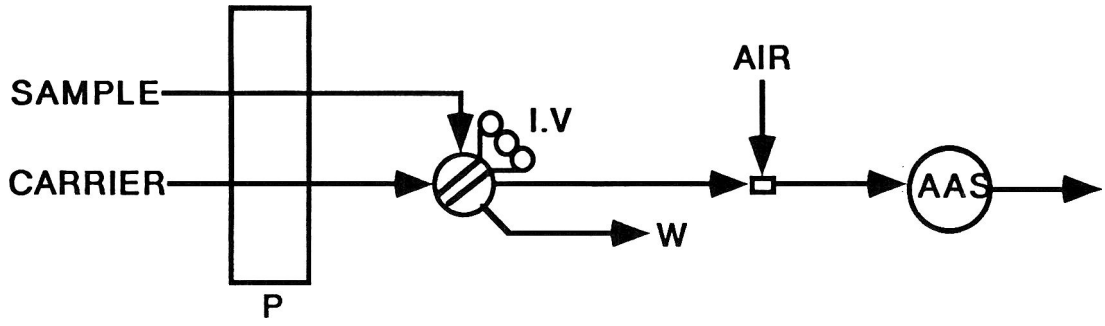

Figure 1. FIA/AAS setup used in the determination of Zn in the blood and serum from patients with CRI: (P) peristalic pump, (I.V.) injection valve, (W) waste, (AIR) flow nebulization compensating, (AAS) atomic absorption spectrometer.

channels are merged via an injection valve. thus permitting the introduction of a fixed volume of sample in the carrier channel and taking the sample directly to the detector. To compensate the carrier flow with the aspiration flow of the nebulizer, an air intake was inserted to ensure correct nebulization of the sample.

Measurement was carried out in the repeated integration mode with a double beam and a deuterium lamp background correction. The optimized parameters are listed in Table 1.

Method 2.—Determination of Zn in Ultrafiltrate Fluid by GF/AAS

Instrumental operating parameters are given in Table 2. The optimized temperature program is shown in Table 3.

Results and Discussion

Method I

The following analytical characteristics were obtained.

Calibration curve.—Within a concentration range between 0.05 and 2.0 ppm, the data obtained fit the following regression straight line:

$$A = 0.002 + 0.173\ [Zn],\ r = 0.999$$

Table 1. Operating conditions for FIA/AAS

Parameter	Setting
Wavelength	213.9 nm
Observation height	12 mm
Air flow rate	12 L/min
Carrier flow rate	2.5 mL/min
Slit width	1 nm
Lamp intensity	5 mA
Acetylene flow rate	1.7 L/min
Total injection volume	260 µL

where A is absorbance, [Zn] is the concentration of Zn in ppm, 0.002 is the ordinate at the origin, and r is the correlation coefficient.

Precision.—To determine precision, 10 determinations on a single sample of serum and another of blood were made. The coefficient of variation (CV) is defined as $CV = s/X_m \times 100$, where s is the standard deviation ($s = 0.002A$ for blood and serum) and X_m is the mean signal ($X_m = 0.038A$ for blood and serum). A CV of 5.3% was obtained for both cases.

Accuracy.—To check the accuracy of the method, the recovery of standard additions of 1.0 ppm Zn in serum and blood was studied. The mean recoveries from 10 samples each of serum and blood were, in both cases, within the 95–105% range.

Detection limit.—To determine the detection limit (DL), 10 determinations of the blank, with a mean value of ($X_m = 0.001A$) and a standard deviation of $0.001A$, were made. DL was defined as $3s/m$ (9), where s is the standard deviation and m is the slope of the calibration curve. A DL of 0.02 ppm was obtained.

Because of reports in the literature (10–11) that do not involve acid digestion of serum and blood, initial assays were performed that involved only dilution and used Triton X-100 as the matrix modifier at different concentrations. Using the maximum dilutions possible so that the analytical signal would

Table 2. Operating parameters for GF/AAS

Parameter	Setting
Wavelength	213.9 nm
Slit width	1 nm
Pyrolytic tube	—
Furnace height	10 mm
Total injection volume	30 µL
Mode peak area	—
Background corrector	D_2 lamp

Table 3. Temperature program used in GF/AAS for determination of Zn in ultrafiltrate fluid

Step	Stage	Temperature, °C	Time, s	Ar flow, L/min
Drying	1	75	5.0	3.0
	2	90	30.0	3.0
	3	105	15.0	3.0
Char	4	500	15.0	3.0
	5	500	2.0	0.0[a]
Atomization	6	1700	1.1	0.0[a]
	7	1700	2.0	0.0[a]
Cleaning	8	1800	2.0	3.0

[a] Stages of signal reading.

be above the detection limit did not significantly reduce the matrix effect. This problem and the fact that many of the samples were not homogenized (having a gelatinous aspect) made it necessary to use acid digestion to obtain a smaller matrix effect and complete homogenization.

The concentrations of Zn in serum and blood were determined by linear calibration and by the standard additions method; in both cases, the results were significantly similar.

Table 4. Concentration of Zn in arterial serum and blood[a]

	Zn, ppm			
	Linear calibration		Standard additions	
Sample	Mean	S_{n-1}	Mean	S_{n-1}
Arterial blood	2.20	0.038	2.15	0.032
Arterial serum	0.46	0.016	0.43	0.025

[a] Comparison of the mean values ($n = 5$) by Student t-test did not reveal significant differences ($p < 0.05$).

Thus, we eliminated the standard additions method and reduced the time and work involved (Table 4).

Method 2

The following analytical characteristics were obtained.

Calibration curve.—The procedure was applied over a concentration range of 0.3–2.0 ppb, and a calibration curve that fits the following regression straight line was obtained:

$$A = 0.004 + 0.075\ [\mathrm{Zn}],\ r = 0.999$$

Table 5. Concentrations of Zn (ppm) in ultrafiltrate fluid, blood, and serum taken from 5 patients with CRI on hemofiltration at different times

Sample	Sampling time, h[a]	Concentration of Zn in indicated patient, ppm					Mean	Amplitude
		1	2	3	4	5		
Ultrafiltrate fluid	1	0.15	0.15	0.16	0.12	0.36	0.188	0.24
	2	0.16	0.16	0.16	0.13	0.38	0.198	0.25
	3	0.16	0.14	0.16	0.12	0.40	0.196	0.28
	4	0.17	0.14	0.19	0.13	0.36	0.198	0.23
Arterial serum	Pred.	0.46	0.43	0.46	0.47	0.69	0.502	0.26
	1	0.51	0.53	0.49	0.50	0.68	0.542	0.19
	2	0.53	0.50	0.43	0.49	0.73	0.536	0.30
	3	0.55	0.57	0.53	0.53	0.73	0.582	0.20
	4	0.53	0.54	0.53	0.66	0.70	0.592	0.17
Venous serum	1	0.53	0.58	0.53	0.60	0.52	0.552	0.08
	2	0.55	0.60	0.51	0.55	0.64	0.57	0.13
	3	0.52	0.55	0.53	0.60	0.66	0.572	0.14
	4	0.51	0.55	0.54	0.66	0.65	0.582	0.15
Arterial blood	Pred.	2.5	3.0	2.2	2.6	2.5	2.56	0.8
	1	2.5	2.5	1.8	2.7	2.4	2.38	0.9
	2	2.5	2.5	1.8	2.7	2.4	2.38	0.9
	3	2.7	2.7	2.4	2.4	2.5	2.54	0.3
	4	2.8	2.8	2.4	3.4	2.6	2.8	1.0
Venous blood	1	2.7	2.8	1.8	2.7	2.7	2.54	1.0
	2	2.9	3.4	2.7	2.5	2.4	2.78	1.0
	3	3.5	3.3	3.4	2.7	3.1	3.2	0.8
	4	3.0	1.8	3.5	3.3	2.8	2.88	1.7

[a] Samples were taken at indicated number of hours after start of hemofiltration. Pred. = predialysis.

Table 6. Two-way ANOVA table

Sample	Source	df	Sum of squares	Mean square	F_{exp}	Significance[a]
Ultrafiltrate fluid	Treatment (A)	3	4.5	1.5	1.69	N
	Repeated measure (B)	3	41.5	13.833	15.56	Y
	AB	9	8	0.889		
Arterial blood	Treatment (A)	4	0.594	0.149	3.72	Y
	Repeated measure (B)	4	1.286	0.322	8.05	Y
	AB	16	0.634	0.04		
Venous blood	Treatment (A)	3	1.122	0.374	1.36	N
	Repeated measure (B)	4	0.175	0.044	0.16	Y
	AB	12	3.293	0.274		
Arterial serum	Treatment (A)	4	0.027	0.007	7.00	Y
	Repeated measure (B)	4	0.155	0.039	3.90	Y
	AB	16	0.022	0.001		
Venous serum	Treatment (A)	3	0.002	0.001	2.00	N
	Repeated measure (B)	4	0.028	0.007	3.50	Y
	AB	12	0.02	0.002		

[a] $p \leq 0.05$: N, no; Y, yes.

where A is absorbance, [Zn] is the concentration of Zn (ppb), and r is the correlation coefficient.

Precision.—From 10 determinations of a single sample, a CV of 5.7% was obtained (CV = S/X_m, $S = 0.005$, $X_m = 0.087$)

Accuracy.—Accuracy was determined from the recovery of standard additions of 1.0 ppb Zn in 10 samples each of serum and blood. In both cases, recoveries in the 93–107% range were obtained.

Detection limit.—Similarly, after 10 determinations of a blank, a mean value ($X_m = 0.004A$) and a standard deviation ($s = 0.003A$) were obtained. A DL of 0.12 ppb was obtained from the same equation for DL used in Method 1.

The samples were diluted in ultrapure water alone and not in acid medium (HNO_3 A.R.), because the amount of zinc in this reagent produces a considerable degree of contamination; therefore, the method loses a certain amount of sensitivity.

The concentration of Zn in the ultrafiltrate fluid was determined by linear calibration and the standard additions method, both giving significantly similar results. Therefore, as for Method 1, the standard additions method can be eliminated to save time and work.

Application of the Methods

The methods were used to determine the concentration of Zn in samples of serum (arterial and venous), blood (arterial and venous), and ultrafiltrate fluid from 5 randomly selected patients with chronic renal impairment on hemofiltration (4 h per session). The samples from the arterial line were taken before and at 1 hour after the start of filtration. For venous blood, venous serum, and ultrafiltrate fluid, samples were collected every hour after hemofiltration started. With this experimental design, our aim was to study whether there were variations in the Zn concentrations in the blood, serum, and ultrafiltrate fluid of patients undergoing hemofiltration, because there are discrepancies in the data reported in the literature (12–14). The experimental results are shown in Table 5.

Interpretation of the analytical data was carried out by analysis of variance (ANOVA). Our experiments responded to a model of paired samples with 2 variation factors, one fixed (times at which the samples were collected) and the other random (patients). This model is, therefore, a mixed model or a random block model without interaction.

For the ultrafiltrate fluid, the data corresponding to patient 5 were somewhat suspect. A test for rejecting extreme observations yielded a $t_{exp} = 0.883$, compared with a t_α with $n = 5$. There is significance at $p < 0.05$, and patient 5 had to be rejected.

As with all statistical techniques, ANOVA is based on certain hypotheses that should be verified for the technique to be considered valid. It is necessary to assume random and paired samples and normality and homogeneity of variances. Normality and homogeneity of variances, however, cannot be strictly checked, because only one observation in each cell (treatment–block intersection) was available. In the case of normality, it is only possible to accept this assumption without checking. To check the homogeneity of variances in a very general way, one can obtain the amplitudes of the sample from each treatment

and accept homogeneity of variances if the discrepancies in the amplitudes are not excessive. The last column of Table 5 shows the amplitudes, and their variations do not appear to be excessive.

Table 6 summarizes the ANOVA data for each sample studied. The F-tests performed ($p \leq 0.05$) suggest that the concentrations of Zn in ultrafiltrate fluid, venous blood, and venous serum do not vary with the times considered, whereas in arterial blood and serum this difference does exist. Additionally, the between-block significance, for all samples studied, shows that Zn concentrations vary among the different patients, although the differences among patients was not of particular interest in our study. However, the between-patient significance furnished important information; planning the experiment in the format of paired samples was suitable.

Conclusions

After setting up both procedures and applying them to the determination of Zn in samples of blood, serum, and ultrafiltrate fluid, the following conclusions may be drawn.

The samples of ultrafiltrate fluid only require dilution in ultrapure water.

The samples of blood and serum require acid digestion to decrease the matrix effect caused by the samples.

It is not necessary to apply the standard additions method in any of the cases.

It is necessary to control the addition of reagents, even those of analytical grade, because they are a source of contamination in the determination of Zn in ultrafiltrate fluid, where Zn concentrations are very low (in the ppb range).

There is no significant increase in Zn levels in ultrafiltrate fluid after hemofiltration.

There are no significant differences in Zn levels in venous blood and serum after hemofiltration.

The Zn concentrations in arterial blood and serum vary during hemofiltration.

The results are consistent with some of data reported in the literature (12–14).

Acknowledgments

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RESIDUES AND TRACE ELEMENTS

Analysis of Flusilazole and Its Major Phenyl Metabolite (IN-F7321) by Approaches Based on Gas Chromatography

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Two basic, previously unpublished procedures were developed to obtain flusilazole data for regulatory purposes. One method involves extraction into ethyl acetate, cleanup on silica or Florisil, and analysis of flusilazole only by packed-column gas chromatography (GC) with nitrogen–phosphorus detection. The second method involves extraction into ethyl acetate, followed by gel permeation chromatography and analysis by capillary GC with mass-selective detection for both IN-F7321 (major metabolite) and flusilazole. Several adaptations of the second procedure increase the analytical options for flusilazole. The parent-only method was applied to analysis of more than 18 matrixes. The parent-plus-metabolite method and its adaptations were applied to the analysis of more than 33 matrixes. Cereals, fruits, animal matrixes and related processed fractions were analyzed. Average recoveries for the parent-only method were generally above 90% (always above 80%) with standard deviations of 12% or less for fortifications ranging from 0.010 to 1.0 ppm. Average recoveries ranged from 84 to 108% for flusilazole and 82 to 111% for IN-F7321 fortifications from 0.010 to 2.0 ppm for the parent-plus-metabolite method. Standard deviations ranged from 3.6 to 20%.

Flusilazole (1-[bis(4-fluorophenyl)(methyl)silylmethyl]-1*H*-1,2,4-triazole) is the active ingredient in numerous Du Pont products such as Punch C fungicide and Nustar fungicide. This compound is effective against powdery mildew, eyespot, rust, net blotch, and rhynchosporium on cereals; apple scab, cedar apple rust, grape black rot, and powdery mildew on apples and grapes; and Stigatoka on bananas. This broad range of applications has led to more than 50 registrations in many countries and the issuance of permanent MRLs (maximum residue levels) by the World Health Organization (WHO) and Food and Agriculture Organization (FAO) Joint Meeting on Pesticide Residues in food (1).

Significant residue data have been generated to support these flusilazole registrations. Parent flusilazole and its major phenyl-containing metabolite, IN-F7321, have been the compounds of most interest to regulators with parent being the most significant residue (Figure 1).

Two basic analytical methods were developed. Method I (parent-only method) is for parent only and Method II (parent-plus-metabolite method) is for simultaneous analysis of flusilazole and IN-F7321. Method II was adapted for many applications. The development and application of the original methods and their adaptations resulted in a menu of options for the analysis of flusilazole and IN-F7321. The following sections will present the original methods and their significant adaptations.

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Experimental

Reagents and Standards

(a) *Standards.*—Flusilazole, 99.9% pure; IN-F7321, <97.3% pure (E. I. du Pont de Nemours and Co., Wilmington, DE).

(b) *Solvents.*—Ethyl acetate and hexane should be equivalent in quality to Fisher Optima Grade. Isopropyl alcohol, methanol, toluene, acetone, and methylene chloride can be LC grade.

(c) *Cleanup packings.*—Adsorbosil silica, 200/425 mesh (Alltech Associates, Inc., Deerfield, IL), Sep-Pak silica cartridges (Waters Associates, Milford, MA); Bio Beads SX-3 (Bio Rad Laboratories, Richmond, CA); Florisil 60/100 mesh (Fisher, Fair Lawn, NJ); LiChroprep Si 60 (40–63 μm) silica gel column (310 mm × 25 mm) (EM Laboratories, Elmsford, NY; Mega Bond Elut, 20 cc, silica-bonded phase (Analytichem International, Harbor City, CA); Phenogel; 5 μm, 50 Å gel permeation column, 30 cm long, 7.8 mm id (Phenomenex, Torrance, CA).

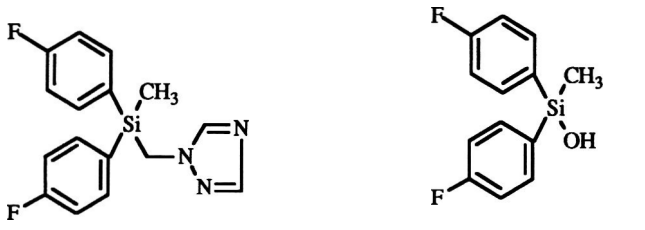
Apparatus

(a) *Homogenizer.*—STD Tissumizer equipped with an SDT-182 EN shaft and generator (Tekmar, Cincinnati, OH).

(b) *Centrifuge.*—Model K floor centrifuge (International Equipment Co., Needham Heights, MA).

(c) *Rotary vacuum evaporator.*—Rotovapor-R (Brinkman Instruments, Westbury, NY), operated under vacuum with the flask in a 35°C water bath.

(d) *Gas chromatograph I (GC I).*—Tracor Model 560.



Flusilazole
(MW = 315)

IN-F7321
(MW = 250)
(hydroxyl replacement of
methylene azole substituent)

Figure 1. Structures of flusilazole and its major metabolite.

(e) *GC I detector.*—Nitrogen–phosphorus detector with air and hydrogen flows at 100 and 2.6 mL/min, respectively. Operating temperature, 300°C.

(f) *GC I column.*—Silanized glass column (2 ft × ¼ in. id), packed with 3% SP2250DB on 100/120 mesh Supelcoport (Supelco, Bellefonte, PA); helium carrier gas, 25–30 mL/min; oven temperature, 180°C; injection port temperature, 248°C. Alternative oven temperature program: initial temperature, 130°C (1 min hold), to 230°C (15 min hold) at 5°C/min.

(g) *Gas chromatograph II (GC II).*—Model 5890 (Hewlett-Packard, Avondale, PA), with a split/splitless injector operated in the splitless mode (split flow, ~20–30 mL/min; purge flow, ~4 mL/min); injection volume, 2 µL; purge flow on at 0.5 min.

(h) *GC II detector.*—Mass-selective detector, 5970 Series (Hewlett-Packard); interface, 280°C; capillary column exit introduced directly into the ion source; electron impact ionization, 70 ev; *m/z* 233 for flusilazole and *m/z* 235 for IN-F7321.

(i) *GC II data system.*—Series 300 ChemStation with a 20-megabyte disk and color monitor (Hewlett-Packard), operating software version 3.1.1.

(j) *GC II column.*—Cross-linked methyl silicone, fused-silica capillary column, 25 m × 0.31 mm id, 0.52 µm film thickness (Hewlett-Packard). Column oven program: 100°C for 0.5 min, programmed to 265°C at 25°C/min, held at 265°C for 15 min. Alternative column, 0.26 mm id, 1.0 µm film, 15 m methyl silcone column (J&W Scientific, Folsom, CA).

(k) *Automated cleanup chromatograph (LC).*—Custom made by using a 16-loop electronically activated injection valve (Valco, Houston, TX) with 2 mL loops, a Valco injection valve for loading, Milton-Roy Model minipump (2-headed) operated at a flow rate of 5 mL/min, and an Isco Foxy fraction collector with accessory controller used for both the LiChroprep Si 60 and gel permeation (Bio Beads SX3) columns.

(l) *Cleanup GPC column.*—Glass (650 mm × 30 mm id) with adjustable plunger ends (ABC Laboratories).

Cleanup Procedures

(a) *Florisil.*—Wash a 25 g amount of Florisil twice with 100 mL hexane–acetone (98 + 2, v/v, with 200 µL H_2O/100 mL mixture) and pack into a reusable glass column (60 cm × 2.5 cm id). Apply a 5 mL sample in hexane plus two 1 mL sample tube hexane rinses to the column. Elute in successive order with 5 mL hexane, 100 mL hexane–toluene (90 + 10, v/v), 100 mL hexane–isopropyl alcohol (93 + 7, v/v), and 200 mL hexane–isopropyl alcohol–methanol (85 + 10 + 5 v/v/v). Collect the final 155 mL eluant, concentrate by rotary evaporation to a few milliliters, transfer to a tube with ethyl acetate rinses, and adjust to a 1 mL final volume under a gentle stream of nitrogen.

(b) *Silica medium-pressure LC cleanup.*—Equilibrate the LiChroprep Si 60 column in hexane–isopropanol–methanol (75 + 15 + 10, v/v/v). Apply a 2 mL sample in hexane to the column (in the automated cleanup LC), discard the first 170 mL volume of eluant, and then collect the next 100 mL. Concentrate the collected volume to a few milliliters on the rotary evaporator, transfer with ethyl acetate rinses to a test tube, and adjust to a final volume of 1 mL with ethyl acetate. The life of the LiChroprep Si 60 column can be extended by putting samples through a Silica Sep-Pak (Waters) prior to the LiChroprep column as follows. Apply a 5 mL sample in hexane to the Sep-Pak (prewashed with 5 mL hexane) along with two 1 mL hexane rinses of the sample tube. Put 10 mL hexane–toluene (90 + 10, v/v) through the Sep-Pak followed by 10 mL hexane–isopropyl alcohol (90 + 10, v/v). Collect the hexane–isopropyl alcohol eluant and reduce the volume to about 0.5 mL under nitrogen and then adjust to 5 mL with hexane. Note that the Sep-Pak eluant is weaker than the LiChroprep Si 60 eluant, a situation that greatly extends the life of the LiChroprep Si 60 column.

(c) *Gel permeation.*—Wash 80 g Bio Beads SX-3 (200–400 mesh) with hexane–ethyl acetate (50 + 50, v/v) to remove fines, and then pack the entire volume of Bio Beads into the column and put the column in the automated cleanup LC. Pump hexane–ethyl acetate (50 + 50, v/v) through the column at 5 mL/min. Apply a 2 mL sample to the column in hexane–ethyl acetate (50 + 50, v/v), void the first 130 mL (26 min), collect the next 80 mL, and then allow the column to pump 90 mL wash before applying the next sample. Reduce the 80 mL collected volume to ~1 mL by rotary evaporation, transfer to a tube with ethyl acetate rinses, and reduce to final volume under a stream of nitrogen. Make all dilutions or adjustments with ethyl acetate.

(d) *Silica.*—Pack 10 g Adsorbosil silica into a 30 cm × 2 cm id glass column in hexane. Apply a sample containing flusilazole and IN-F7321 in 6 mL hexane (5 mL sample, 2 × 0.5 mL sample tube rinses). Put 90 mL hexane–isopropyl alcohol (95 + 5, v/v) through the cartridge and elute the first 40 mL to waste (collect the second 50 mL; this contains IN-F7321). Put 60 mL hexane–propanol–methanol (85 + 10 + 5, v/v/v) through the silica and collect (contains flusilazole). Reduce the collected fractions to a final volume of 1 mL; use ethyl acetate for adjustments and rinses.

(e) *Gel permeation–LC.*—Inject 100 µL sample (in ethyl acetate) onto 2 phenomenex Phenogel gel permeation columns (in series; flow rate, 1.5 mL/min; mobile phase, ethyl acetate). Discard the first 14.25 mL eluant, collect the next 4.5 mL eluant, and then discard another 15 mL as a sample wash.

(f) *Silica Mega Bond Elute.*—Place a silica Mega Bond Elute on a vacuum manifold and wash twice with 5 mL hexane–ethyl acetate (50 + 50, v/v). Apply a 1 mL sample and allow migration onto the cartridge without vacuum. Elute the cartridge with 5 mL hexane–ethyl acetate (50 + 50, v/v) and collect the eluant (no vacuum).

(g) *Silica Sep-Pak.*—Apply 2 mL sample in hexane and two 0.5 mL sample tube hexane rinses to a silica Sep-Pak (prewashed with 15 mL ethyl acetate followed by two 10 mL hexane volumes). Elute the Sep-Pak batchwise as follows: 10 mL 30% methylene chloride–70% hexane (elute to waste), 12 mL 3% isopropyl alcohol–97% hexane (collect, contains IN-F7321), and 12 mL 2% methanol–5% isopropyl alcohol–93% hexane (collect, contains flusilazole).

Sample Prepreparation (Methods I and II)

Grind samples while frozen (dry ice may be necessary) to a fine powder or chop appropriate sample fractions (i.e., apple quarters from each apple in a sample) to fine pieces. Keep the preprocessed samples frozen until analysis.

Analytical Procedure

(a) *Method I.*—Extract 50 g samples [apples (EA), grapes (EA), cereal grains (EA), cereal straw (EA), cereal forage (EA), peanut meats (H), peanut shells (H), sugar beets (EA), banana (EA), peaches (H)] with 150–200 mL the specified solvent (EA, ethyl acetate; H, hexane) with the homogenizer and a 250 mL polypropylene centrifuge bottle. Cereal, apple, and grape samples must include 20 mL 0.1 N NaOH added before the organic solvent. Centrifuge at ~4700 × *g* for 10 min and decant the organic solvent to a 500 mL evaporator flask. Add another 150–200 mL solvent to the centrifuge bottle and repeat the homogenization, centrifugation, and decanting steps (combine extracts). Concentrate all extracts to ~100–200 µL (rotary evaporator to ~1–2 mL, transfer to tube with ethyl acetate rinses, reduce under a stream of nitrogen, and then adjust to exactly 5 mL with hexane). Put apple and grape samples through the Florisil cleanup. Put cereal grain, cereal straw, cereal forage, peanut shell, sugar beet, banana, and peach samples through the LiChroprep Si 60 column. Evaporate all solvent from peanut meat extracts (rotary evaporator) and dilute the remaining oil (15–30 mL) to 50 mL with hexane and extract twice with 100 mL methanol. Reduce the combined methanol extracts to ~200 µL, dilute to 5 mL with hexane, and put through the LiChroprep Si 60 column cleanup. Prepare standards in ethyl acetate.

(b) *Method II.*—Extract 5 g apple, grape, cow muscle, cow liver, cow kidney, cow fat, milk, cereal grain, cereal straw, cereal forage, or whole plant samples twice with 150 mL ethyl acetate (add 20 mL 1N NaOH for apples, grapes, grain, and straw) with the homogenizer, centrifuge (~4700 × *g*), and decant the organic solvent. Evaporate the solvent to near dryness and adjust to 2 mL with ethyl acetate. Add 2 mL hexane and clean up by gel permeation chromatography (*Cleanup Procedures*, part c) before analysis by gas chromatography (GC) with a mass spectrometric detector (GC II). Prepare standards in control matrix processed as specified for the samples. Serial

Table 1. Flusilazole recovery data and residue values for actual treatments with analyses by packed-column gas chromatography with nitrogen–phosphorus detection (Method I)[a]

	Fortifications, ppm			Analysis of treated samples		
Matrix	Range, ppm	No. of samples	Average recovery ± SD, %	Locations (PHI range, days)	Varieties (treatment rate range, oz ai/acre)	Residue range, ppm
Apples	0.010–1.0	15	100 ± 7.6	4 (44–72)	5 (0.25–2.0)	<0.010–0.058
Grapes	0.010–1.0	10	98 ± 7.3	1 (49)	1 (0.25–1.0)	<0.010–0.044
Raisins and raisin waste	0.20–0.50	4	98 ± 7.3	2 (28–31)	1 (0.50–2.0)	<0.010–0.44
Wheat grain	0.010–1.0	9	101 ± 6.4	1 (65)	1 (2.0–4.0)	<0.010–0.010
Wheat straw	0.010–1.0	13	95 ± 7.2	1 (65)	1 (2.0–4.0)	<0.026–0.17
Wheat forage	0.030–1.0	11	95 ± 4.9	1 (0–21)	1 (2.0–8.0)	0.31–40.0
Peanut (meats)	0.010–0.10	54	93 ± 8.6	19 (7–83)	3 (0.25–4.0)	<0.010–0.22
Peanut (shells)	0.010–1.0	37	94 ± 8.2	13 (7–130)	3 (0.25–8.0)	<0.010–8.1
Peanuts (whole)	0.010–0.10	9	97 ± 8.9	4 (12–83)	2 (0.25–4.0)	<0.010–0.075
Pressed peanut meal	0.010–0.050	8	94 ± 11	3 (19–38)	1 (2.0–4.0)	<0.010–0.012
Peanut oil	0.030–0.50	16	91 ± 7.6	3 (19–38)	1 (2.0–4.0)	0.033–0.090
Peanut soapstock		13	90 ± 9.2	3 (19–38)	1 (2.0–4.0)	<0.020–0.062
Sugarbeet roots	0.010–0.10	8	82 ± 7.3	—	—	—
Banana (pulp)	0.010–0.50	31	91 ± 8.9	14 (1–28)	1 (1.0–2.9)	<0.010–0.032
Banana (peel)	0.010–2.0	30	90 ± 11	14 (1–28)	1 (1.0–2.9)	<0.010–0.13
Peaches	0.010–0.10	10	93 ± 8.8	1 (2–7)	1 (0.5–2.0)	0.13–0.77
Pears	0.010–0.50	3	83 ± 12	2 (91–98)	1 (0.5–5.0)	<0.010

[a] Abbreviations: SD, standard deviation; oz ai, ounces of active ingredient.

additions to a single control (with proper volume adjustments) can be used for manual injections.

Adaptations of Method II

Method II was adapted by the originators and by several analytical facilities. These adaptations have led to a menu of analytical options.

(a) *Adaptation A.*—Reduce the volume of the gel-permeation-collected fraction to ~200 µL and then adjust to 5 mL with hexane. Put the sample through the silica cleanup and analyze the flusilazole fraction on the packed-column GC (GC I). Analyze the IN-F7321 fraction on the capillary GC system (GC II) on a J&W fused-silica 0.26 mm id, 1.0 µm film, 15 m methyl silicone column. All standards are prepared in ethyl acetate rather than control matrix.

(b) *Adaptation B.*—Insert a plug of nonsilanized glass wool into the injection port liner. Condition the liner–glass wool extensively with matrix and prepare all standards in ethyl acetate. Remove the liner occasionally, wash with organic solvents (glass wool insert intact), and reuse. Use methylene chloride to prepare and elute the GPC column and then clean up the GPC eluant (taken to dryness and redissolved in 2 mL hexane) by the silica Sep-Pak procedure in (b) of the *Cleanup Procedures* section above. Analyze by the GC II system using an HP-17, 25 m × 0.2 mm id, 0.17 µm film column (column temperature program: 100°C for 0.8 min, 45°C/min to 190°C, 35°C/min to 235°C, and then 30°C/min to 265°C; inlet, 210°C).

(c) *Adaptation C.*—Reduce the sample extract to ~0.2 mL and adjust to a 4 mL volume (2 mL hexane, and 2 mL ethyl actate). Put a 1 mL aliquot into a 10 mL tube and add 200 µL MSTFA [*N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide]; seal the tube and react at room temperature for at least 30 min. Evaporate the mixture to ~0.1 mL under a stream of nitrogen at room temperature and then adjust the volume to 1 mL with hexane. Clean up the sample on a silica Mega Bond Elut, adjust to a final volume of 1 mL, and analyze. Monitor the silyated IN-F7321 with *m/z* 307.

(d) *Adaptation D.*—Reduce the sample extract (2 g sample) to ~0.2 mL, adjust to 2 mL with ethyl acetate, and filter through a 0.2 µm low-volume filter. Clean up a 100 µL aliquot of the filtered sample by gel permeation–LC. Reduce the collected fraction to ~0.1 mL and dilute to 2 mL with hexane. Clean up the sample on a silica Sep-Pak. Reduce the volumes of the collected fractions to 1 mL for IN-F7321 and 250 µL for flusilazole and analyze with separate injections.

Calculations

Calculations are done with external standards in 1 of 3 ways. A standard curve is generated from standards interspersed with the samples during analyses, and analyte concentrations are calculated from a linear regression formula. Alternatively, a standard curve is run before samples are injected, and the curve is repeated periodically throughout the sample analyses with calculations done by linear regression from a calibration curve constructed from bracketing standard curves. In addition, residues can be calculated by using an average response factor determined from bracketing standards around individual samples.

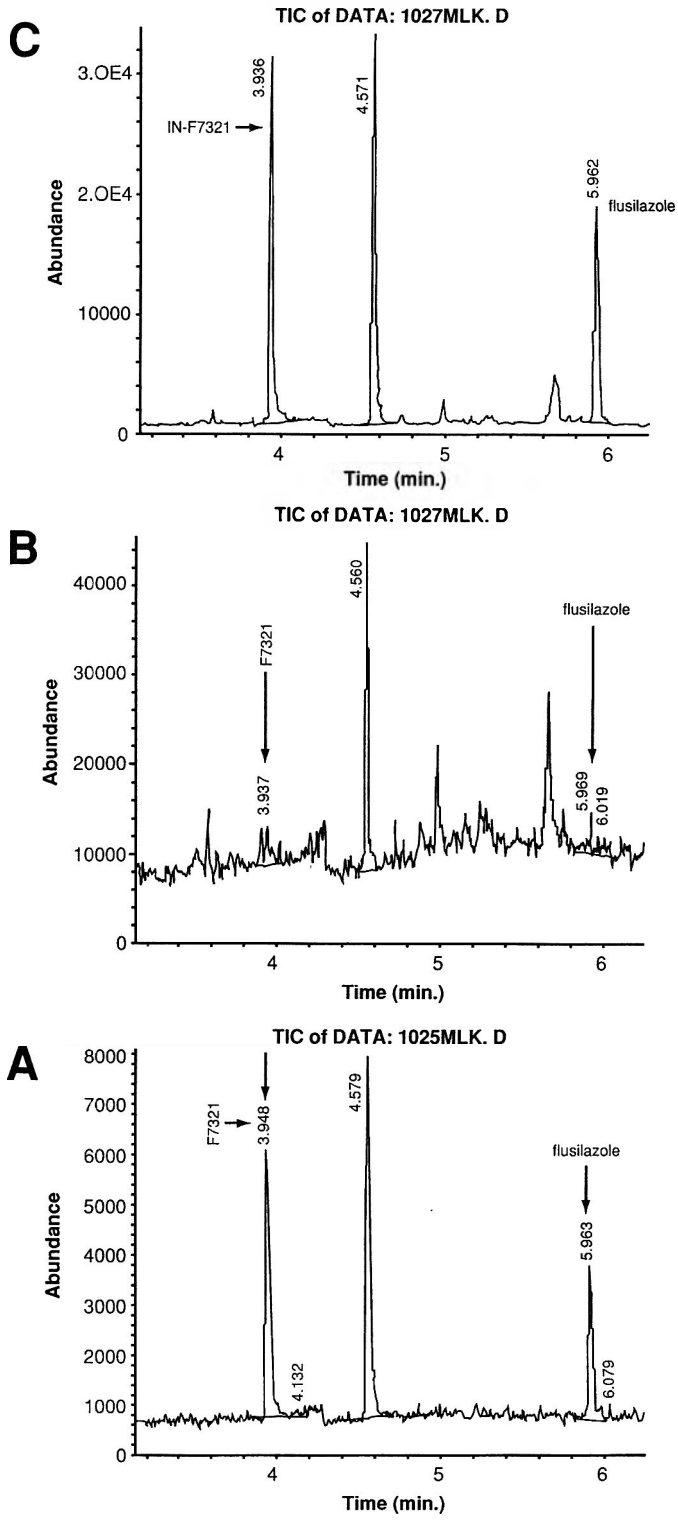


Figure 2. Typical Method I chromatograms for analysis of grapes by packed-column gas chromatography with nitrogen–phosphorus detection (oven temperature program for GC I). A, 0.1 µg/mL flusilazole; B, control grape; C, 0.010 ppm control grape fortification.

Table 2. Recovery data and residue values for actual treatments with analyses by the unadapted and adapted Du Pont GC-MS method (Method II)

	Fortifications[a]				Analysis of treated samples			
			Average recovery ± SD, %				Residue range, ppm	
Matrix	Range, ppm	No. of samples	Flusilazole	IN-F7321[b]	Locations (PHI range, days)	Varieties[c] (treatment rate range, oz ai/acre)	Flusilazole	IN-F7321[b]
Apples	0.010–1.0	26	88 ± 20	97 ± 17	21 (7–14)	7 (1.0–2.0)	<0.010–0.33	<0.010–0.10
Apple process fractions (wet pomace, dry pomace, juice)	0.010–0.40	6	84 ± 9.8	87 ± 18	2 (7)	2 (2.0)	<0.010–0.40	<0.010–0.77
Grapes[d]	0.010–0.10	34	84 ± 17	87 ± 15	27 (7–31)	8 (0.05–1.0)	<0.010–0.16	<0.010–0.060
Grape process fraction[d] (wet pomace, dry pomace, juice)	0.020–0.50	6	93 ± 17	92 ± 22	2 (14–28)	2 (1.0–2.0)	<0.010–0.13	<0.010–0.45
Raisins and raisin waste[d]	0.040–0.30	5	95 ± 14	82 ± 14	1 (14)	1 (1.0–2.0)	0.09–0.61	<0.07–0.67
Cow milk, skim milk, cream[e]	0.020–0.20	43	106 ± 19	89 ± 19	1 (–1 to 28 days of feeding)	1 (2.0–50 ppm in feed)	<0.010–0.080	0.010–0.70
Cow muscle[e]	0.010–0.20	10	87 ± 17	84 ± 15	1 (–1 to 28 days of feeding)	1 (2.0–50 ppm in feed)	<0.010–0.020	<0.010–0.17
Cow liver[e]	0.020–0.40	10	94 ± 20	94 ± 18	1 (–1 to 28 days of feeding)	1 (2.0–50 ppm in feed)	<0.010–0.81	<0.010–0.28
Cow fat[e,f]	0.020–0.40	18	87 ± 13	87 ± 16	1 (–1 to 28 days of feeding)	1 (2.0–50 ppm in feed)	<0.010–0.050	<0.010–0.92
Cow kidney[e]	0.040–0.50	19	108 ± 19	90 ± 12	1 (–1 to 28 days of feeding)	1 (2.0–50 ppm in feed)	<0.010–0.020	<0.050–5.0
Wheat, rye, barley, grain	0.010–0.050	13	87 ± 20	83 ± 9	17 (52–94)	(2.3–3.6)	<0.010–0.020	<0.010–0.020
Wheat, rye, barley, straw	0.010–0.99	24	86 ± 18	78 ± 5	20 (49–94)	(2.3–3.6)	0.040–1.8	0.090–0.45
Wheat, rye, barley, ears	0.010–0.20	14	79 ± 14	74	16 (0–56)	(2.3–3.6)	<0.010–6.9	0.020–0.46
Wheat, rye, barley, stalks	0.010–1.1	8	77 ± 11	101 ± 7.8	16 (0–56)	(2.3–3.6)	0.040–2.2	0.040–0.38
Wheat, rye, barley, green plant	0.010–2.0	31	86 ± 17	89 ± 12	19 (0–56)	(2.3–3.6)	<0.010–6.5	<0.010–0.39

[a] Fortifications were done simultaneously with flusilazole and IN-F7321, usually at the same concentration.
[b] Few IN-F7321 fortifications (1-4 per matrix) or analyses not considered a regulatory issue after limited scouting analyses.
[c] Varieties unknown for cereals; winter, spring, and summer barley; winter, spring, and summer wheat; and winter rye analyzed.
[d] Analyzed with the unadapted method (standards in control matrix).
[e] Analyzed with extensive injection port conditioning and a multiramp GC oven program.
[f] Omental, subcutaneous, and renal fats were analyzed separately.

Table 3. Recovery data for apples and grapes analyzed by the adaptation where the analytes are separated on silica and analyzed separately with standards in ethyl acetate[a]

Matrix	Range, ppm	No. of samples	Average recovery ± SD, %	
			Flusilazole	IN-F7321
Apples	0.010–0.20	9	96 ± 11	107 ± 11
Grapes	0.010–0.10	12	102 ± 8.6	92 ± 11
Apple and grape dry pomace	0.050–1.3	7	95 ± 14	111 ± 3.6
Raisins and raisin waste	0.030–0.50	12	103 ± 14	87 ± 16

[a] Analysis of flusilazole by packed-column GC with nitrogen–phosphorus detection; analysis of IN-F7321 by GC/MSD.

Results and Discussion

Method I

Flusilazole can be analyzed efficiently by extraction, cleanup on some absorbent, and analysis by packed-column GC with nitrogen–phosphorus detection (Method I). Table 1, showing both fortification and sample analysis data, establishes the applicability of the method. Average recoveries are generally above 90% (always above 80%) with standard deviations of 12% or less for fortification levels ranging from 0.010 to 1.0 ppm for 12 matrixes. The analysis of 16 field-treated crops or their processed fractions showed the applicability to actual analyses. Figure 2 shows chromatograms for a grape analysis. This grape chromatography is typical for worst case background from Method I-type analyses. The column oven temperature program option is used to help resolve interferences.

Method II

Concurrent analysis of flusilazole and IN-F7321 is most conveniently accomplished by GC with mass-selective detection (GC/MS). This can be done after a simple, automated, gel permeation chromatography cleanup. The 2 main advantages of this approach are the simple workup for 2 analytes and the relatively clean chromatograms. Two disadvantages associated with the capillary chromatography column and splitless injection are (*1*) a matrix enhancement effect that essentially doubles the response of the analyte in matrix over the same concentration in neat solution and (*2*) the higher standard deviations (as high as 20%) for fortification recovery data (Table 2). The easiest way to handle the matrix effect is to prepare standards in control matrix. This amounts to standard addition, which is a classical analytical technique.

The matrix effect can be managed (*1*) by extensive and frequent conditioning of an injection port liner that contains an unsilanized glass wool plug; (*2*) by putting the sample through silica, analyzing flusilazole by packed-column GC (as in Method I), and analyzing a separate silica elution fraction by GC/MS for IN-F7321; (*3*) by using a more efficient cleanup by gel premeation–LC followed by a silica Sep-Pak cleanup; and (*4*) by silylating the sample followed by clean up on a Mega BondElut. Analysis after the extensive injection port conditioning uses a multiple-ramp GC oven program. The amount of conditioning required is matrix dependent and could range from several hours of injection to a day of injections. The first 2 solutions to the matrix enhancement effect require more work and handling and reduce sample throughput. The second 2 solutions do not require more work but do require a derivatization or an LC pump.

Recovery data and actual sample analysis (from treated field crops or flusilazole-fed cows) for application of Method II are given in Table 2 (includes standards prepared in matrix and standards in neat solution with extensive injection port conditioning). Average recoveries ranged from 77 to 108% (flusilazole) and from 74 to 101% (IN-F7321) for 20 matrixes. Standard deviations ranged from 5 to 22% and are typical of splitless injections. Table 3 shows recovery data for separate analyses of flusilazole and IN-F7321 after chromatography on silica (Method II, Adaptation A). Recoveries ranged from 95 to 103% for flusilazole (standard deviation, 8.6 to 14%) and 87 to 111% for IN-F7321 (standard deviation, 3.6 to 16%). Table 4 shows recovery data for the analysis of grapes by Adaptation C and of apples by Adaptation D. Recoveries averaged 87 ± 14% (flusilazole) and 85 ± 14% (IN-F7321), for grapes and 103 ± 15% (flusilozole) and 95 ± 16% (IN-F7321) for apples.

Figure 3 shows a representative chromatogram for analysis of flusilazole and IN-F7321 by GC with mass-selective detection. The chromatograms are from whole milk, which is important to regulators and contains a lot of fat. Fat and oil must be eliminated sufficiently to allow consistent chromatography when analyzing many samples. Chromatographic background is usually not a problem with mass-selective detection.

Table 4. Recovery data for analysis of flusilazole and IN-F7321 by Method II, adaptations C and D

Crop	Fortification		Average recovery ± SD, %			
			Adaptation C		Adaptation D	
	Range, ppm	No. of samples	Flusilazole	IN-F7321	Flusilazole	IN-F7321
Grapes	0.020–0.20	29	87 ± 14	85 ± 14	—	—
Apples	0.010–0.030	27	—	—	103 ± 15	95 ± 16

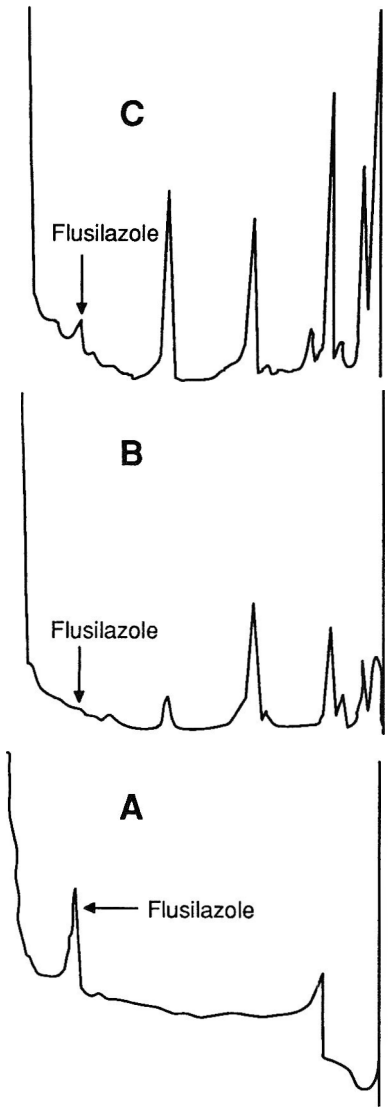


Figure 3. Typical Method II chromatograms for analysis of flusilazole and IN-F7321 in whole milk. Note the different y-axis scales on each chromatogram (autoscaling of y-axis to largest peak). A, 0.05 mg/mL flusilazole and IN-F7321 (equivalent to a 0.010 ppm residue); B, whole-milk control; C, 0.050 ppm control whole-milk fortification.

Extraction Efficiency

Ethyl acetate extraction is predominantly used for these analytical procedures. When the ethyl acetate extractions specified above are used on samples from plant and animal metabolism studies (actual field treatments or fed animals) an average of 88% of total radioactivity present in the tissues is extracted (range, 62–100%). Table 5 shows the actual matrixes and percentages involved. Considering that flusilazole is readily metabolized to IN-F7321 and then to conjugates and bound residues, the extraction efficiencies indicated in Table 5 are quite good and validate the efficient performance of the method.

In addition, a field-treated, post-ethyl acetate extraction grape sample was put through extensive reflux extraction by procedures used to study grape metabolism of flusilazole. Reanalysis of the extensively extracted sample showed no new flusilazole or IN-F7321. The original residue method ethyl acetate extraction showed actual residues for the sample studied. This result indicates that flusilazole and IN-F7321 were efficiently removed by the residue method extraction.

Solvent Consumption

Multiple extractions followed by preparative cleanup steps (Method I and II and Adaptations A and B of Method II) can consume significant amounts of solvent. Adaptations C and D of Method II consume less solvent, using approximately 315 and 427 mL/sample, respectively. Adaptation D could be adjusted so that smaller samples (2 gm) could be extracted twice with 20 mL organic solvent each time and still maintain the solvent/sample ratio established in Method II. This would reduce the solvent consumption to approximately 131 mL/sample.

Table 5. Ethyl acetate extraction of radiolabeled (^{14}C) flusilazole-produced residues from various plant and animal matrices (validation of extraction efficiency)

Matrix	Percent of total radioactivity extracted[a]
Apples	82
Chicken eggs	95
Chicken breast muscle	98
Chicken thigh muscle	98
Goat loin muscle	92
Chicken liver	62[b]
Goat liver	88
Chicken kidney	83
Chicken fat	100
Goat milk	95
Goat milk	86
Wheat straw	76
Average	88

[a] Percent of total radioactivity present in tissues after treatment or feeding with ^{14}C flusilazole.
[b] Suspected large number of nonextractable, bound metabolites.

Conclusion

Flusilazole and its major metabolite (IN-F7321) can be analyzed effectively in many matrixes by a variety of analytical options. The options can be chosen to fit particular analytical and regulatory needs. Analysis of flusilazole alone can be accomplished with extraction, a single cleanup step, and packed-column analysis with nitrogen–phosphorus detection.

When it is necessary to analyze IN-F7321, the parent and metabolite are quantitated by capillary GC with mass-selective detection. If controls are available, the standards can be prepared in control matrix. If controls are not available, extensive injection port conditioning, silica cleanup, or derivatization steps followed in some cases by separate analyses for flusilazole and IN-F7321 can be used.

Acknowledgment

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Reference

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RESIDUES AND TRACE ELEMENTS

Preparation of Spiked Soils by Vapor Fortification for Volatile Organic Compounds Analysis

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This paper describes a vapor fortification method for preparing quality assurance/quality control soils for volatile organic compound analysis. Treatment of soils with volatile organic compounds occurs in a closed container in a manner somewhat analogous to the way the vadose zone often becomes contaminated. One advantage of this method for preparing soils for quality assurance/quality control purposes is that the efficiency of various extraction methods can be reliably compared. Furthermore, by substantially reducing the error due to sample inhomogeneity, the error associated with the determinative step can also be properly evaluated.

The wide use and subsequent improper disposal of volatile organic compounds (VOCs) have made this group of chemicals our most common environmental hazardous waste problem (1–3). Nevertheless, no readily available source of performance evaluation materials exists that provides quality assurance/quality control (QA/QC) for the analysis of VOCs in soil (2). Attempts to spike, homogenize, and transfer soils have proven unsatisfactory because of the inability to control volatilization losses (4). Presently, the estimation of analytical VOC accuracy relies on the results of sample spike-and-recovery tests. Usually, this evaluation method involves addition of the VOCs of interest diluted in methanol (MeOH) to the purge vessel of a purge-and-trap system after introducing the test sample, which may either be "blank" (unfortified) water or an aqueous–soil suspension. In either case, this method of evaluating performance focuses only on the determinative step, allows little time for the analytes to interact with the soil, and does not simulate the manner in which soils become contaminated in the field.

Vapor fortification offers an alternative means for creating test soils containing VOCs that circumvents all of these problems (5–9). An earlier feasibility study assessed the parameters requiring special attention, producing a protocol that minimized the influences of treatment duration, laboratory temperature and relative humidity, and composition of the treatment solution (8). The vapor fortification method of spiking is analogous to the process by which vadose zone soils are contaminated by vapors from liquid pools, and avoids introducing either large quantities of water or MeOH into the test matrix.

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Vapor-fortified soils have been used to determine both extraction efficiency and analytical accuracy, thus providing a comprehensive method for assessing a laboratory's capability (6, 7). However, because this method avoids liquid–solid contact, it may fail to be representative of soils that have experienced long-term exposure to organic solutions.

Experimental

Materials

The text matrix used was the standard reference soil from the U.S. Army Environmental Center (formerly the U.S. Army Toxic and Hazardous Materials Agency). This material is a marine sediment comprised mainly of uniform medium-grain sand with ca a 10% silt–clay fraction and an organic carbon content of 0.91%. Test analytes selected were *trans*-1,2-dichloroethylene (TDCE), trichloroethylene (TCE), benzene (Ben), and toluene (Tol). These analytes are often found at hazardous waste sites and were used in previous studies (1–3, 6–9). A stock solution for vapor fortification, which was also used to prepare analytical standards, was prepared monthly by taking the following approximate analyte quantities to volume in 100 mL of MeOH: 0.60 g Tol, 0.59 g TCE, 0.50 g TDCE, and 0.35 g Ben. This stock solution was diluted as necessary in MeOH, then combined 1:1 (v/v) with tetraethylene glycol dimethyl ether (tetraglyme) to create the 50 mL fortification solution used for soil treatments. All of the chemicals were reagent grade quality or better.

Soil Preparation, Treatment, and Handling

The test soil was air-dried, sieved through a 30–mesh screen, and thoroughly mixed before subsampling. Soil subsamples weighing ca 2.00 ± 0.01 g were accurately weighed in 1.0 mL glass ampules and placed in a 2.6 L desiccator containing $CaSO_4$ desiccant. After 2 days of desiccation, the desiccant was removed and replaced by an open 60 mL glass bottle containing 50 mL of fortification solution. Thus, the open ampules containing soil subsamples were treated in the closed desiccator by exposure to the vapor from a 50 mL solution containing MeOH, tetraglyme, and the analytes of interest.

After 7 or more days of treatment, the chamber was opened and glass beads 5 mm in diameter were rapidly placed on top of each of the ampules, forming temporary seals. Then, as quickly as possible, each ampule was put in a metal tension clamp, the neck of the ampule was pinched just below the glass bead, and the ampule heat-sealed, leaving a sharp point, by using a propane plumbers torch.

Table 1. Percents of analyte absorption into soil, normalized to a 7-day exposure[a]

	Days of exposure						
Compound	1	2	3	5	7	9	11
TDCE	81	102	111	96	100	99	95
Ben	64	81	91	93	100	102	100
TCE	66	84	95	91	100	100	100
Tol	54	75	85	91	100	103	104

[a] Percents represent mean of duplicate samples.

Table 2. Concentrations of fortification solutions vs. measured soil concentrations (concentrations in μg/g)[a]

	Dilution of Stock MeOH Solution		
Compound	1:1	1:10	1:100
TDCE	8.1	0.79	0.093
Ben	8.9	0.94	0.10
TCE	10	1.0	0.12
Tol	12	1.2	0.14

[a] Soil concentrations each represent the mean of 6 determinations.

Analysis

Ampules were placed tip down in 40 mL volatile organic compound analysis (VOA) vials containing 30 mL of water and were broken by vigorous shaking of the vials by hand. Headspace portable gas chromatographic (GC) analysis was performed after extracting the VOCs into the 30 mL of Type 1 water contained inside a sealed 40 mL VOA vial (6–10; Spittler, T.M., U.S. Environmental Protection Agency, Environmental Services Division Region 1, Lexington, MA personal communication, 1989). The VOA vial was equipped with an open-faced cap and Teflon-lined silicone rubber septum through which headspace samples were withdrawn after 2 min of vigorous shaking by hand. Due to material strengths, only high-quality VOA vials should be used, and gloves should be worn while breaking the ampule and dispersing the soil. Alternatively, when following Method 8240 (11), the sealed ampules are opened inside of VOA vials containing MeOH or equipped with a modified purge-and-trap adapter (Associated Design Model PT-6005-0002) for purge-and-trap GC/mass spectrometric analysis. Peak height responses of samples were measured from strip-chart recordings to 2 significant figures.

Results and Discussion

Table 1 shows the rate of VOC uptake by this soil matrix for these particular treatment parameters. The results shown in this table are for the analysis of duplicate subsamples removed from the exposure chamber after 1, 2, 3, 5, 7, 9, and 11 days of exposure and have been expressed as the percent analyte absorbed, normalized to day 7. Intermittent opening of the desiccator allows moisture from the room air to enter and interfere with absorption, i.e., compete for active sites on the desiccated soil (8). For this reason, the concentration maxima observed are not representative of the levels obtainable when the desiccator remains shut during the critical uptake period. As demonstrated previously, it appears that once a particular soil sample attains a maximal concentration of a VOC, that sample remains stable (with respect to gain or loss of VOC) in the desiccator for up to 30 days (6).

Table 3. Analyte concentrations (μg/g) established for sets of triplicate samples vs holding time and fortification batch[a]

	Holding time			
Compound	1 day	28 days	60 days	120 days
TDCE	8.0 ± 0.3	8.0 ± 0.3	8.2 ± 0.9	8.1 ± 0.9
Ben	8.8 ± 0.3	9.1 ± 0.3	9.3 ± 0.4	8.9 ± 0.4
TCE	10 ± 0.6	11 ± 0.6	11 ± 0.6	11 ± 0.5
Tol	12 ± 0.6	12 ± 0	12 ± 0.6	13 ± 0.6
	Batches			
Compound	A	B	C	D
TDCE	8.0 ± 0.2	8.2 ± 1.1	7.8 ± 0.4	7.9 ± 0.3
Ben	8.8 ± 0.3	8.4 ± 0.3	8.7 ± 0.3	8.3 ± 0.4
TCE	10 ± 0.6	11 ± 0.6	10 ± 0.1	11 ± 0.6
Tol	12 ± 0.6	12 ± 0	11 ± 0.6	11 ± 1.0

[a] Concentrations are expressed as mean plus or minus the standard deviation.

To assess the relationship between the concentration of the treatment solution and the concentration of the fortified matrix, test samples were exposed to treatment mixtures containing 25, 2.5, and 0.25 mL of the stock solution (Table 2). The results show that the measured concentrations approached values that would have been predicted based on simple dilution of the stock standard (Henry's Law) (see Table 2). This range in soil concentrations of VOC, approximately 10 μg/g to 0.1 μg/g, encompassed the action levels typically used to guide site investigators in the treatment of contaminated soils.

To be useful for site investigation applications, fortified soils for performance evaluation must be both reproducible and stable for time periods in excess of 14 days and preferably for months to years. To evaluate both batch-to-batch precision and holding time vs stability, mean concentrations were compared using an analysis of variance (ANOVA) at the 95% confidence level. All sealed ampules were stored at room temperature. Statistical analysis failed to establish significant differences in analyte concentration either among treatment batches (A, B, C, and D) or holding periods (1, 28, 60, and 120 days) (Table 3).

On the basis of the results, vapor fortification shows promise as an alternative means of producing VOC-treated soils for performance evaluation purposes. The sample handling protocol that was established results in good precision among triplicates, with relative standard deviations typically ≤5%.

Summary

The advantage of using vapor fortification as a method of preparing performance evaluation samples, as opposed to the methods of direct injection of either aqueous or methanolic solutions, is that it allows for evaluation of both the extraction process and of instrumental precision (6). The efficiency of an extraction method is often attributed to the influence that organic matter in the soil has on the partition coefficients and is particularly important with regard to extracting the more hydrophobic VOCs (12–15). However, this phenomenon has not been addressed by methods that create performance evaluation samples just prior to analysis. Vapor fortification provides samples for performance evaluation that can be used both to certify portable GC methods for aqueous extraction that are suitable for on-site analysis as well as to test standard laboratory protocols.

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RESIDUES AND TRACE ELEMENTS

Packed-Column Gas Chromatographic Method for the Simultaneous Determination of 10 Pyrethroid Insecticide Residues in Fruits, Vegetables, and Grains

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A simple, rapid, packed-column gas chromatographic method was developed for simultaneous determination of 10 pyrethroid insecticide residues (allethrin, biphenthrin, cyhalothrin, permethrin, cyfluthrin, flucythrinate, fenvalerate, fluvalinate, deltamethrin, and py-115) in fruits, vegetables, and grains. These multiresidues are extracted from various crops with acetone–petroleum ether and cleaned up on a Florisil column (for the fruits and vegetables) or on a Florisil–charcoal–alumina column (for the grains) prior to their determination by gas chromatography with an electron capture detector. Recoveries of 10 pyrethroid compounds from 12 different crops (maize, soybeans, wheat, sorghums, paddy, potatoes, cucumbers, cauliflowers, spinaches, apples, bananas, and oranges) fortified at levels of 0.02–5.00 ppm ranged from 58 to 130%. In a separate precision study, coefficients of variation were 5.5–14.6% at 0.1–0.5 ppm (n = 10, maize), and 4.1–12.1% at 0.010–0.050 ppm (n = 10, apples). The detection limits of the method ranged from 2.0 to 10.0 ppb on a crop basis.

The synthetic pyrethroids are a newly developed group of insecticides that has gained worldwide attention because of the greater photostability, enhanced insecticidal activity, and relatively low toxicity of these compounds compared with organochlorine and organophosphorus insecticides. Early in the 1980s, our country began to research and to use this type of pesticide. Presently, there are 10-odd varieties being used in growing grains, fruits, and vegetables. At the same time, many countries around the world conducted a great deal of research on the residual effect of these compounds on the environment after use. Now, the Food and Agriculture Organization (FAO) and World Health Organization (WHO) have prescribed residue limits for some pyrethroids in agriculture and livestock products (1).

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Methods for the analysis of residues of individual pyrethroid compounds in a variety of crops (2–4) have been presented and reviewed, e.g., the determination of allethrin, cyhalothrin, cypermethrin, deltamethrin, fenpropathrin, fenvalerate, fluvalinate, permethrin, phenothrin, resmethrin, and tetramethrin. Two types of multiresidue procedures have also been developed. The first type is the comprehensive multiresidue method, which is useful for detecting organophosphorus- or organohalogen-containing pesticide residues as well as pyrethroids (5, 6). The other type focuses on the simultaneous determination of pyrethroid multiresidues, the subject of discussion in this paper. Published methods of this second type include determining 3 to 6 pyrethroid residues (cypermethrin, permethrin, cyhalothrin, deltamethrin, biphenthrin, fenpropathrin, and tetramethrin) in fruits, vegetables, and grains (7–10). Until now, only 6 pyrethroid residues have been reported to be determined simultaneously by packed-column gas chromatography (GC) (11, 12). In addition, it is interesting to note that an analytical procedure, based on an AOAC Official Method (13), is being studied. The intent is to incorporate the pyrethroid residue procedure into the AOAC multiresidue method, but a final conclusion has not been obtained.

In this paper, we describe a modification of an existing AOAC method for the simultaneous determination of the 10 pyrethroids in different crops. The proposed method uses acetone–petroleum ether to extract pyrethroid residues from various crops. The extracts from fruits and vegetables are passed through a Florisil column, while those from grains and oily crops are passed through a mixed column of Florisil–charcoal–alumina. The purified extracts are analyzed using packed-column GC with electron capture detection.

Experimental

Apparatus

(a) *GC system.*—(*1*) Shimadzu Model GC-14A (Shimadzu Corp., Kyoto, Japan) equipped with ^{63}Ni electron capture de-

tector (ECD) and with a 2.0 m × 3.2 mm id glass column, packed with 5% SE-30 on 60–80 mesh Shimalite W.AW-DMCS. Operating conditions: injector port, 280°C; column, 255°C; ECD, 300°C; carrier gas (N_2), flow 75 mL/min. (*2*) Hewlett-Packard Model 5890A (Hewlett-Packard, Palo Alto, CA) equipped with ^{63}Ni ECD and with a 1.8 m × 2 mm id glass column packed with 3% OV-1 on 100–120 mesh Chromosorb W-HP. Operating conditions: injector port, 300°C; column, 240°C; ECD, 300°C; carrier gas (N_2), flow 20 mL/min.

(**b**) *Chromatographic cleanup column.*—30 cm × 2 cm id glass column fitted with stopcock, and equipped with 100 mL funnel top: (*1*) *Florisil column.*—Place small plug of glass wool at the bottom of the column and add 1 cm layer of anhydrous Na_2SO_4. Introduce 4 g Florisil into the column and tap sides of the column to produce even packing. Top with 1 cm layer of anhydrous Na_2SO_4. Prewash column with 30 mL ethyl acetate–petroleum ether (5 + 95). (*2*) *Mixed column.*—Place small glass-wool plug at the bottom of the column and add 1 cm layer of anhydrous Na_2SO_4. Pour in column in the following order: 2 g Florisil, 0.3 g activated charcoal, and 5 g alumina (neutral). Tap the sides of the column to produce even packing. Add 1 cm layer of anhydrous Na_2SO_4. Prewash column with 30 mL dichloromethane–ethyl acetate–petroleum ether (35 + 10 + 55).

(**c**) *High speed blender.*—Model DC-200 (Shanghai Model Tool Factory, Shanghai, China).

(**d**) *Mechanical shaker.*—Model HY-2 with variable movement control (Jiangsu Changzho Guohua Instrument Factory, Jiangsu, China).

(**e**) *Homogenizer.*—Model T25 (Janke & Kunkel-IKA-Labortechnik, Staufen, Germany).

(**f**) *Centrifuge.*—Type Z320 with 200 mL centrifuge tube (Berthold Hermle GmbH & Co., Laboratory Centrifuges, Gosheim, Germany).

(**g**) *Rotary vacuum evaporator.*—Model RE-51 rotary evaporator (Yamato Scientific Co., Ltd., Tokyo, Japan).

Reagents

Analytical reagent grade materials were used unless otherwise indicated.

(**a**) *Solvents.*—Dichloromethane, ethyl acetate, acetone, petroleum ether (at 30–60°C) (Beijing Chemicals Factory, Beijing, China). Redistill in all-glass apparatus and check by GC.

(**b**) *Anhydrous sodium sulfate.*—Heat at 600°C for 24 h and then cool in desiccator (Beijing Chemicals Factory).

(**c**) *Activated charcoal.*—20–40 mesh (Beijing Guanghua Wood Factory, Beijing, China). Reflux with 1 M HCl for 4 h, wash to neutral pH with water, and place in an oven at 95–100°C until it attains a constant weight. Store in sealed container at room temperature.

(**d**) *Alumina (neutral).*—80–100 mesh (Shanghai Five-Four Chemical Reagent Factory, Shanghai, China). Activate at 300°C for 4 h and then cool in desiccator.

(**e**) *Florisil.*—60–10 mesh, Baker Analyzed Reagent (J.T. Baker, Inc., Phillipsburg, NJ). Heat at 150°C for 24 h before use. Store in sealed container at room temperature.

(**f**) *Pesticides.*—Deltamethrin (98%), Roussel-Uclaf Nanjing Office (Nanjing, China); cypermethrin (97%), Shell China Ltd. (Beijing, China); biphenthrin (94.3%), FMC Far East Ltd. (Beijing, China); cyhalothrin (97.0%), ICI Agrochemicals (Beijing, China); permethrin (91.1%), allethrin (92.3%), tetramethrin (94.6%), fenpropathrin (92.3%), and fenvalerate (94.1%), Sumitomo China Chemical Ltd (China); flucythrinate (94.2%), fluvalinate (90.6%), cyfluthrin (93.8%), and s-5439 (94.7%), Shanghai Midwest Pesticide Factory (Shanghai, China); methothrin (95.0%) and py-115 (93.7%), Yang Zhou Pesticide Factory (China). Prepare individual standard solutions in *n*-hexane at 100–500 μg/mL concentration. Prepare standard cumulative solutions (for recovery study) by mixing suitable volumes of individual standard solutions and diluting with *n*-hexane.

Extraction of Pesticide Residues from Various Crops

Weigh 20.0 g chopped sample into high-speed blender jar, add 100 mL petroleum ether–acetone mixture (1 + 1) and blend for 2 min at high speed. Filter the mixture through a Buchner funnel by suction. Rinse blender jar with two 20 mL portions of the above described mixed solvents, and use the washings to rinse the residues in the Buchner funnel. Transfer the filtrate to a 500 mL separating funnel, and wash the Buchner flask with two 10 mL portions of the above described mixed solvents. Combine these with the extract. Wash organic extract with two 100 mL portions of 2% aqueous NaCl (w/v), discarding aqueous phase in the lower layer each time. Remove traces of water by slowly passing organic extract through glass-funnel containing glass wool plug and ca 15 g anhydrous sodium sulfate. Collect the filtrate into a 250 mL round-bottom flask. Rinse the separating funnel with 20 mL petroleum ether. Pass rinsings through the same funnel containing anhydrous sodium sulfate and collect them in the round-bottom flask with the organic extract. Evaporate contents of the flask to ca 5 mL volume on a rotary evaporator at 40°C.

Chromatographic Column Cleanup

Elution pattern for each lot of adsorbent must be predetermined.

(**a**) *Fruits and vegetables.*—Transfer the concentrated extract from the round-bottom flask onto the previously packed Florisil column, and let the solution percolate into the column. Rinse the round-bottom flask with 2 additional 5 mL portions of petroleum ether and add the washings to the column. Elute pyrethroid residues with 50 mL ethyl acetate–petroleum ether mixture (5 + 95), collecting the eluate in a 250 mL round-bottom flask. Carefully evaporate eluate fractions just to dryness with rotary evaporator at 40°C and redissolve in 10.0 mL petroleum ether.

(**b**) *Grain and oily crops.*—Transfer one-half of the concentrated extract from the round-bottom flask onto the previously packed mixed column and let the solution percolate into the column. Rinse the round-bottom flask with 2 additional 5 mL portions of petroleum ether and add the washings to the column. Elute pyrethroid residues with 70 mL of a dichloromethane–ethyl acetate–petroleum ether mixture (35 + 10 +

55), collecting eluate in a 250 mL round-bottom flask. Evaporate eluate fractions just to dryness with a rotary evaporator at 40°C and redissolve in 20.0 mL petroleum ether.

Results and Discussion

Compared with determinations of organophosphate and carbamate insecticides, the determination of pyrethroid insecticide residues is more difficult because the majority of the 15 compounds included in our experiment contain a cyclopropane ring that is similar in function to a double bond. These rings may cause the formation of stereoisomers, with certain pyrethroid compounds forming 8 stereoisomers (14). In fact, most of the pyrethroid insecticides are a mixture of different stereoisomers. Not only can the various pyrethroids be separated, but also the stereoisomers of the same pyrethroid can be resolved when being analyzed by packed-column chromatography. The aim of this paper is for the 15 different pyrethroid insecticides to be separated from each other as much as possible and for the stereoisomers of each pyrethroid to be resolved very little or not at all.

(a) *Appraisal of chromatographic behaviors of the 15 pyrethroid residues tested.*—A comparative test on the chromatographic behaviors of 15 pyrethroid insecticides has been performed with 13 types of packed columns as well as with 2 different instruments in 2 laboratories. The results are partially shown in Table 1 and Table 2. The data has fully demonstrated that the column polarity, ratio of stationary liquid phase, and column temperature are the 3 major factors that influence the separation of pyrethroids. As seen in Table 1, these 15 pyrethroid compounds belong to the same category of insecticide, but their behaviors on 13 types of packed columns differ substantially. Therefore, the polarity ranges of these compounds is wide. For example, with nonpolar packed columns such as 5% SE-30, 10 pyrethroid compounds are separated in about 7 min, and the different stereoisomers of each pyrethroid appear in a single peak. If one wishes to resolve the stereoisomers of the individual pyrethroid compounds, polar packed columns such as 3% OV-25 and 2% XE-60 may be selected. However, none of these 10 pyrethroid compounds are separated with the 10% DEGS packed column within 20.0 min. Highly polar packed columns, such as DEGS and Carbowax 20M, are not suitable for determination of pyrethroid residues. The results also indicate that 7 of the above mentioned 13 columns may be used for determination of individual pyrethroid residues. In terms of multiresidue analysis, the optimized results have shown that both 5% SE-30 and 3% OV-1 are found to be the best packed columns for simultaneous determination of 10 out of 15 pyrethroids. The best chromatographic conditions are described. As for the remaining 5 pyrethroid insecticides, methothrin does not show sufficient sensitivity. The other 4 pesticides (biphenthrin, fenpropathrin, tetramethrin, and s-5439) cannot be well-resolved from each other under the optimized GC conditions, but may be resolved with a 3% OV-25 column. The ratio of stationary liquid phase has a large effect on the separation of pyrethroid compounds. It takes 10.4 min for 10 pyrethroids to be completely resolved on 1.5% OV-17, whereas only 8 of 10 pyrethroids were separated in 32.0 min on 10% OV-17 and the pattern was unsatisfactory. Take the retention time of allethrin as an example: 0.47 min at 255°C on the 1.5% OV-17 but 4.0 min at 270°C on the 10% OV-17. The retention times differ by a factor of 10 without regard to the effect of temperature. The effects of the column temperature and the carrier gas flow on the separation of the pyrethroid residues are shown in Table 2. One effect of column temperature is that it has a strong impact on retention time. For example, the retention time of deltamethrin was 47.67 min at 225°C when the flow rate of the carrier gas was 60 mL/min on 1.5% OV-17. However, it was 5.15 min at 275°C when the other conditions are not changed, the difference in retention times being 10-fold.

(b) *Choice of extraction and cleanup conditions.*—A number of solvent systems are satisfactory for residue analysis of the individual pyrethroids. Because the chemical structure of pesticides is extremely similar, the use of a common procedure for extraction and cleanup is deemed feasible. After comparative testing and optimization of the various extraction and cleanup systems (9, 11, 12, 14, 15), we have submitted a modified AOAC method for the determination of 10 pyrethroid residues in various crops, in which an acetone–petroleum ether mixture is selected as a common extraction system for fruits, vegetables, and grains. In addition, a new Florisil–charcoal–alumina column is developed for purification of extracts from various crops. (*1*) *Samples of fruits and vegetables.*—Take ethyl acetate–petroleum ether (5 + 95) as the eluant and make a comparative test for the 3 chromatographic cleanup columns: the 4 g Florisil column, the 4 g magnesia column, and the 1 g activated charcoal column. The best separation of the 10 pyrethroids was obtained with the first column. The next best separation was obtained with the magnesia column. With regard to the activated charcoal column, most of allethrin and py-115 could be eluted off, but the remaining 8 compounds cannot be recovered. Take 2 activated charcoal columns previously eluted with ethyl acetate–petroleum ether (5 + 95), and one column was eluted with dichloromethane, the other with methanol. The result indicated that most of pyrethroids in the column were quantitatively eluted with dichloromethane, while elution with methanol resulted in no improved yields. Based on the results of the comparison of the 3 cleanup columns, the Florisil column was chosen for the proposed method. The proportion of ethyl acetate in the eluant was important. If higher than 5%, the impurities could not be removed; if less than 5%, the recoveries remained extremely low. The procedure prescribes 4–5% as the proper proportion. (*2*) *Samples of grains and oils.*—In accordance with purification procedures for samples of fruits and vegetables, 2 cleanup columns were used, the first consisting of 2 g Florisil, 0.3 g activated charcoal, and 5 g neutral alumina, and the other consisting of 2 g silica gel, 0.3 activated charcoal, and 5 g neutral alumina. The recoveries of the 10 pyrethroids were tested, with 50 and 80 mL, respectively, of ethyl acetate–petroleum ether (10 + 90) as the eluant. None of the results were satisfactory. When elution was performed subsequently with 70 mL dichloromethane–ethyl acetate–petroleum ether (35 + 10 + 55), the results were better, and the purification efficiency of the first

Table 1. Retention times (min) of 15 pyrethroid insecticide residues on 13 types of packed chromatographic columns at selected temperatures with N_2 flow rates

Pesticide	3% OV-1			3% OV-25				3% OV-225, 250°C, 35 mL/min	1.5% OV-17 + 1.95% OV-210, 230°C, 30 mL/min
	240°C, 19.5 mL/min	250°C, 14.4 mL/min	260°C, 14.0 mL/min	250°C, 14.0 mL/min	250°C, 21.0 mL/min	260°C, 21.0 mL/min	270°C, 21.0 mL/min		
Allethrin	1.75	1.65	1.38	2.02	1.57	1.27	1.07	1.06	2.27
Biphenthrin	4.43	3.85	3.01	5.8	4.5	3.35	2.58	2.36	6.13
Fenpropathrin	4.55	3.93	3.08	7.5	5.9	4.18	3.27	4.97	8.46
Tetramethrin	4.19	3.64	3.05	—	6.13	—	—	5.18	8.67
s-5439	4.20	3.65	3.05	—	4.67	—	—	5.22	8.71
	—	—	—	—	6.37	—	—	—	—
Cyhalothrin	5.9	5.05	3.80	4.97	3.9	2.83	2.26	5.56	11.99
	—	—	—	7.98	6.2	4.36	3.56	6.07	—
	—	—	—	8.55	6.7	4.65	—	—	—
Permethrin	7.7	6.57	4.87	14.95	11.7	8.17	5.98	5.3	13.77
	—	—	—	14.3	—	—	—	5.9	15.05
Cypermethrin	—	8.6	6.4	12.9	10.1	7.17	5.3	6.16	—
	—	—	—	20.7	16.2	11.35	8.2	12.89	—
Fenvalerate	13.5	11.1	8.0	20.7	16.2	11.2	8.2	8.83	—
	—	—	—	29.4	22.9	15.5	11.1	17.23	—
	—	—	—	32.2	25.2	16.9	12.0	—	—
Fluvalinate	15.4	12.5	8.9	11.7	9.1	6.36	4.81	—	—
	—	—	—	24.2	18.9	12.55	9.1	—	—
	—	—	—	25.5	19.9	13.02	—	—	—
Deltamethrin	17.4	14.1	10.0	40.0	31.2	20.36	14.6	—	—
	—	—	—	—	33.9	21.97	15.7	—	—
py-115	1.32	1.21	1.02	1.51	1.12	—	—	—	—
Cyfluthrin	9.3	8.4	6.1	10.3	8.1	5.8	4.3	—	—
	9.6	—	—	17.6	13.7	9.4	6.7	—	—
	—	—	—	—	—	—	—	—	—
Flucythrinate	10.8	8.9	6.7	—	—	—	—	—	—
	11.5	9.3	7.0	—	—	—	—	—	—
Methothrin[a]	—	—	—	—	—	—	—	—	—

[a] No response signal.

Table 1. (*Continued*)

Pesticide	2% OV-101, 260°C, 60 mL/min	5% SE-30, 255°C, 75 mL/min	1.5% OV-17, 255°C, 75 mL/min	1.5% OV-17 +3.5% QF-1, 240°C, 75 mL/min	2% XE-60, 250°C, 75 mL/min	10% OV-17, 270°C, 21 mL/min	10% OV-225, 240°C, 16 mL/min	10% OV-101, 250°C, 14 mL/min	10% DEGS[b], 170°C, 17.4 mL/min
Allethrin	1.48	0.78	0.47	1.01	0.70	4.0	7.0	5.7	—
Biphenthrin	2.74	1.85	1.49	2.49	1.28	11.4	18.3	15.1	—
Fenpropathrin	—	—	—	—	—	14.3	—	20.1	—
Tetramethrin	—	—	—	—	—	—	—	—	—
s-5439	—	—	—	—	—	—	—	—	—
	—	—	—	—	—	—	—	—	—
Cyhalothrin	3.48	2.41	2.28	4.78	3.54	16.8	—	27.0	—
	—	—	—	—	—	—	—	—	—
	—	—	—	—	—	—	—	—	—
Permethrin	4.36	3.12	3.67	5.89	2.55	22.8	—	—	—
	—	—	—	—	—	—	—	—	—
Cypermethrin	—	—	—	—	—	26.5	—	—	—
	—	—	—	—	—	—	—	—	—
Fenvalerate	7.14	5.29	7.39	10.97	8.47	32.0	—	—	—
	—	—	—	—	9.5	—	—	—	—
	—	—	—	—	—	—	—	—	—
Fluvalinate	—	5.88	6.89	13.43	11.63	—	—	—	—
	—	—	—	—	—	—	—	—	—
	—	—	—	—	—	—	—	—	—
Deltamethrin	8.95	6.67	10.42	14.39	10.73	—	—	—	—
	—	—	—	—	12.11	—	—	—	—
py-115	1.24	0.60	0.31	0.77	0.61	—	—	—	—
Cyfluthrin	5.17	3.73	4.55	9.15	6.11	25.4	—	—	—
	—	—	—	—	6.87	—	—	—	—
	—	—	—	—	7.41	—	—	—	—
Flucythrinate	—	4.24	5.41	10.75	8.15	—	—	—	—
	—	4.47	5.90	11.44	9.03	—	—	—	—
Methothrin[a]	—	—	—	—	—	—	—	—	—

[a] No responsive signal.
[b] No peak after injecting for 20 min.

Table 2. The effects of temperature and N_2 flow rate on retention time (min) of 15 pyrethroid compounds (1.5% OV-17 Shimalite W.AW-DMCS (201D) 80–100M, 2 m × 3 mm id)

	Column temp, °C																	
	275			265			255			245			235			225		
	N_2 flow rate, mL/min																	
Pesticide	90	75	60	90	75	60	90	75	60	90	75	60	90	75	60	90	75	60
py-115	0.16	0.17	0.19	0.20	0.23	0.27	0.27	0.31	0.36	0.34	0.41	0.48	0.50	0.56	0.62	0.69	0.79	1.31
Allethrin	0.23	0.24	0.28	0.30	0.34	0.40	0.42	0.47	0.54	0.53	0.64	0.74	0.78	0.88	0.97	1.11	1.26	1.44
Methothrin	0.29	0.31	0.36	0.40	0.43	0.51	0.54	0.60	0.69	0.69	0.82	0.95	1.02	1.14	1.26	1.56	1.66	1.89
	0.41	0.44	0.51	0.56	0.61	0.72	0.76	0.87	1.01	1.02	1.22	1.41	1.55	1.22	1.34	2.10	1.77	2.02
	—	—	—	—	—	—	—	—	—	—	—	—	—	1.74	1.91	—	2.56	2.93
Biphenthrin	0.69	0.72	0.84	0.95	1.04	1.22	1.35	1.49	1.74	1.80	2.15	2.49	2.79	3.14	3.44	4.20	4.73	5.42
Fenpropathrin	0.86	0.93	1.07	1.20	1.31	1.54	1.70	1.89	2.19	1.29	2.72	3.15	3.54	3.98	4.36	5.34	6.01	6.90
Tetramethrin	0.92	0.96	1.12	1.28	1.40	1.65	1.83	2.04	2.37	2.49	2.97	3.44	3.90	4.39	4.79	5.95	6.69	7.69
s-5439	0.92	0.96	1.12	1.28	1.40	1.65	1.83	2.04	2.37	2.49	2.97	3.44	3.90	4.39	4.79	5.95	6.69	7.69
Cyhalothrin	1.00	1.05	1.23	1.42	1.55	1.83	2.06	2.28	2.66	2.83	3.37	3.90	4.49	5.05	5.50	6.95	7.81	8.97
Permethrin	1.58	1.66	1.96	2.28	2.48	2.91	3.30	3.67	4.26	4.60	5.44	5.94	6.92	7.69	8.40	10.51	11.87	13.63
	—	—	—	—	—	—	—	—	—	—	—	6.31	7.29	8.20	8.97	10.33	12.75	14.65
Cyfluthrin	1.89	1.99	2.34	2.77	3.01	3.55	4.10	4.55	5.29	5.80	6.88	7.98	9.39	10.58	11.61	14.94	15.75	18.08
	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	16.80	19.30
Cypermethrin	2.22	2.33	2.77	3.25	3.62	4.12	4.20	5.34	6.19	7.11	8.07	8.80	11.09	11.34	13.75	16.76	18.48	21.37
	—	—	—	—	—	—	—	—	—	—	—	9.33	—	12.06	14.15	17.65	19.83	22.91
Flucythrinate	2.24	2.34	2.77	3.30	3.60	4.19	4.90	5.41	6.27	6.96	8.20	9.52	11.36	12.71	14.11	18.18	20.34	23.45
	2.39	2.50	2.96	3.56	3.89	4.54	5.31	5.90	6.84	7.62	9.02	10.45	12.51	14.08	15.53	20.16	22.70	26.12
Fluvalinate	2.76	2.89	3.42	4.13	4.49	5.26	6.21	6.89	8.03	9.52	10.39	12.04	15.37	16.20	17.89	23.41	26.14	30.07
	—	—	—	—	—	—	—	—	—	—	11.15	12.97	—	17.75	19.13	24.07	27.74	31.96
Fenvalerate	3.05	3.19	3.77	4.49	4.88	5.73	6.30	7.39	8.58	9.52	11.15	12.97	15.37	17.75	19.13	24.67	27.74	31.96
Deltamethrin	4.17	4.34	5.15	6.23	6.78	7.94	9.38	10.42	12.08	13.80	16.07	18.65	22.54	25.34	28.26	39.77	41.33	47.67

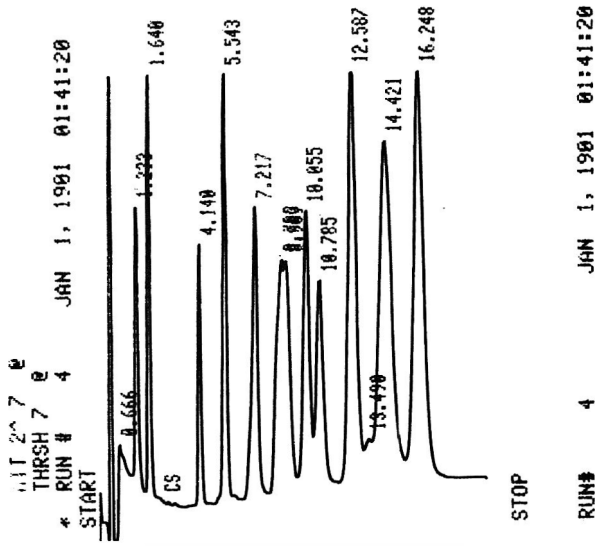

Figure 1. ECD chromatogram of 2 μL injection of a mixed standard: (1) 0.10 ng py-115, (2) 0.10 ng allethrin, (3) 0.20 ng biphenthrin, (4) 0.14 ng cyhalothrin, (5) 0.70 ng cypermethrin, (6) 0.30 ng cyfluthrin, (7, 8) 0.40 ng flucythrinate, (9) 0.36 ng fenvalerate, (10) 0.36 ng fluvalinate, (11) 0.36 ng deltamethrin. 3% OV-1 on 100–120 mesh Chromosorb W-HP, 6 ft. × 2 mm id glass column at 240°C.

column was far superior to the second. Fenvalerate, fluvalinate, and deltamethrin were poorly recovered with the second column. Therefore, the first column was chosen for the proposed method with a mixture of dichloromethane–ethyl acetate–petroleum ether (35 + 10 + 55) as the eluant.

With this method, 10 pyrethroid residues have been determined simultaneously in samples of various fruits, vegetables, and grains. Typical chromatograms obtained from a standard mixture and from a fortified sample using 2 different packed columns are shown in Figures 1 and 2. Except for flucythrinate (whose stereoisomers seemed to be resolved on the 5% SE-30 column, while these isomers were resolved as 2 peaks on the 3% OV-1 column), all the other pyrethroids appeared as a single peak. All 10 of these compounds were easily quantified. Various crops analyzed using this method contained no interfering compounds. Between allethrin and biphenthrin in Figure 2, there was a small peak at a retention time of about 1 min, but it did not interfere with detection of any of the 10 pyrethroids. Recoveries of 10 pyrethroid insecticides from fortified grains and oily crops and from fortified fruits and vegetables are given in Tables 3 and 4, respectively. Recoveries of pyrethroid residues from 10 crop samples fortified at levels between 0.02 and 5.00 ppm ranged from 58 to 130%. Precision data are shown in Table 5, where coefficients of variation ranged from 5.5 to 14.6% for maize (n = 10) and from 4.1 to 12.1% for apples (n = 10). The linear response of the electron capture detector ranged from 0.005 to 3.0 ng for the 10 pyrethroids. The practical determination limit for the whole method is in the range of 2.0–10.0 ppb for the 10 pesticides, based on a 20 g fruit or vegetable samples and a 5 μL injection taken

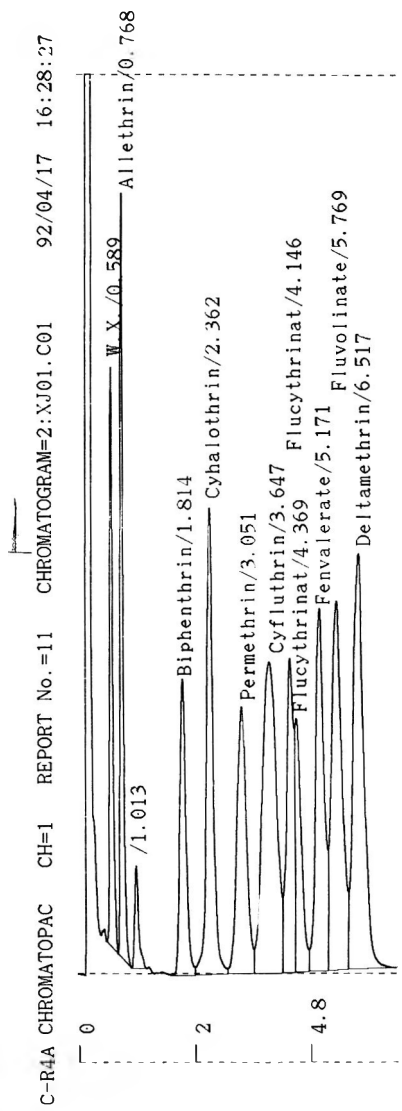

Figure 2. ECD chromatogram of paddy extract fortified with (1) WX (equal to py-115) at 0.10 ppm, (2) allethrin at 0.10 ppm, (3) biphenthrin at 0.14 ppm, (4) cyhalothrin at 0.10 ppm, (5) permethrin at 0.50 ppm, (6) cyfluthrin at 0.16 ppm, (7) flucythrinate at 0.30 ppm, (8) fenvalerate at 0.20 ppm, (9) fluvalinate at 0.20 ppm, (10) deltamethrin at 0.20 ppm. 5% SE-30 on 60–80 mesh Shimalite W. AW-DMCS, 2 m × 3.2 mm id glass column at 255°C. Injected amount: 5 μL extract, equivalent to 2 mg sample.

from a 10 mL final extract volume. In the case of grain samples, a 10 g aliquot was used; therefore, the determination limit was 2 times higher. The results have fully demonstrated that the proposed method is simple, rapid, and accurate and is suitable for multiresidue pyrethroid analysis.

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Table 3. Recovery of pyrethroid insecticides from fortified grains and oily crops

Compound	Maize			Wheat			Sorghum			Paddy			Soybean		
	Added, ppm	Found, ppm	Rec., %	Added, ppm	Found, ppm	Rec., %	Added, ppm	Found, ppm	Rec., %	Added, ppm	Found, ppm	Rec., %	Added, ppm	Found, ppm	Rec., %
py-115	1.00	1.05	105	1.00	1.14	114	1.00	0.92	92	1.00	0.85	85	1.00	0.87	87
	0.100	0.990	99	0.100	0.105	105	0.100	0.097	97	0.100	0.094	94	0.100	0.111	111
	0.020	0.024	119	0.020	0.022	110	0.020	0.019	95	0.020	0.026	130	0.020	0.024	120
Allethrin	1.00	1.15	115	1.00	1.01	101	1.00	0.89	89	1.00	1.01	101	1.00	0.87	87
	0.100	0.942	94	0.100	0.103	103	0.100	0.092	92	0.100	0.098	98	0.100	0.096	96
	0.020	0.026	130	0.020	0.021	105	0.020	0.019	95	0.020	0.017	85	0.020	0.017	85
Biphenthrin	1.40	1.44	103	1.40	1.27	91	1.40	1.11	79	1.40	1.26	90	1.40	1.12	80
	0.140	0.129	92	0.140	0.130	93	0.140	0.130	93	0.140	0.120	86	0.140	0.122	87
	0.028	0.029	103	0.028	0.028	100	0.028	0.023	82	0.028	0.024	86	0.028	0.024	86
Cyhalothrin	1.00	1.03	103	1.00	0.88	88	1.00	0.79	79	1.00	0.89	89	1.00	0.81	81
	0.100	0.088	88	0.100	0.089	89	0.100	0.093	93	0.100	0.081	81	0.100	0.087	87
	0.020	0.019	95	0.020	0.021	105	0.020	0.016	80	0.020	0.017	85	0.020	0.016	80
Permethrin	5.00	5.10	102	5.00	4.40	88	5.00	2.90	58	5.00	3.60	72	5.00	3.27	65
	0.500	0.416	83	0.500	0.468	93	0.500	0.437	87	0.500	0.329	66	0.500	0.401	80
	0.100	0.099	99	0.100	0.072	72	0.100	0.058	58	0.100	0.071	71	0.100	0.063	63
Cyfluthrin	1.60	1.63	102	1.60	1.41	88	1.60	1.17	73	1.60	1.24	90	1.60	1.19	74
	0.160	0.135	84	0.160	0.149	94	0.160	0.140	87	0.160	0.131	82	0.160	0.134	84
	0.032	0.034	108	0.032	0.031	96	0.032	0.026	81	0.032	0.030	94	0.032	0.025	78
Flucythrinate	3.00	3.18	106	3.00	2.58	86	3.00	2.37	79	3.00	2.76	92	3.00	2.55	85
	0.300	0.260	87	0.300	0.279	93	0.300	0.276	92	0.300	0.280	93	0.300	0.342	114
	0.060	0.073	110	0.060	0.059	98	0.060	0.070	116	0.060	0.058	97	0.060	0.055	92
Fenvalerate	2.00	2.01	101	2.00	1.68	84	2.00	1.46	73	2.00	1.80	90	2.00	1.46	73
	0.200	0.166	83	0.200	0.191	96	0.200	0.172	86	0.200	0.164	82	0.200	0.181	91
	0.040	0.048	120	0.040	0.043	108	0.040	0.037	93	0.040	0.035	87	0.040	0.030	75
Fluvalinate	2.00	2.04	102	2.00	1.72	86	2.00	1.30	65	2.00	1.62	81	2.00	1.38	69
	0.200	0.178	89	0.200	0.171	86	0.200	0.169	85	0.200	0.149	75	0.200	0.151	76
	0.040	0.037	93	0.040	0.033	83	0.040	0.031	78	0.040	0.035	88	0.040	0.030	75
Deltamethrin	2.00	1.96	98	2.00	1.64	82	2.00	1.46	73	2.00	1.84	92	2.00	1.44	72
	0.200	0.172	86	0.200	0.182	91	0.200	0.176	88	0.200	0.166	83	0.200	0.168	84
	0.040	0.041	103	0.040	0.045	113	0.040	0.037	93	0.040	0.036	90	0.040	0.029	73

Table 4. Recovery of pyrethroid insecticides from fortified fruits and vegetables[a]

	Cucumber			Spinaches			Cauliflower			Apple			Orange		
Compound	Added, ppm	Found, ppm	Rec., %	Added, ppm	Found, ppm	Rec., %	Added, ppm	Found, ppm	Rec., %	Added, ppm	Found, ppm	Rec., %	Added, ppm	Found, ppm	Rec., %
Py-115	1.00	0.88	88	1.00	0.87	87	1.00	0.94	94	1.00	0.84	84	1.00	1.08	108
	0.100	0.103	103	0.100	0.112	112	0.100	0.092	92	0.100	0.084	84	0.100	0.082	82
	0.020	0.021	105	0.020	—	—	0.020	0.020	100	0.020	0.021	105	0.020	0.016	80
Allethrin	1.00	0.88	88	1.00	0.96	96	1.00	1.01	101	1.00	0.86	86	1.00	1.10	110
	0.100	0.106	106	0.100	0.101	101	0.100	0.085	85	0.100	0.083	83	0.100	0.093	93
	0.020	0.020	100	0.020	0.019	95	0.020	0.015	75	0.020	0.014	70	0.020	0.016	80
Biphenthrin	1.40	1.22	87	1.40	1.18	84	1.40	1.20	86	1.40	1.37	98	1.40	1.43	102
	0.140	0.138	99	0.140	0.140	100	0.140	0.127	91	0.140	0.126	90	0.140	0.134	96
	0.028	0.028	100	0.028	0.028	100	0.028	0.032	114	0.028	0.024	84	0.028	0.025	90
Cyhalothrin	1.00	0.88	88	1.00	0.84	84	1.00	0.84	84	1.00	0.98	98	1.00	1.03	103
	0.100	0.103	103	0.100	0.097	97	0.100	0.086	86	0.100	0.094	94	0.100	0.095	95
	0.020	0.020	100	0.020	0.019	95	0.020	0.019	95	0.020	0.016	80	0.020	0.012	60
Permethrin	5.00	4.49	90	5.00	4.20	84	5.00	3.35	67	5.00	4.87	97	5.00	4.74	95
	0.500	0.519	104	0.500	0.521	104	0.500	0.316	63	0.500	0.515	103	0.500	0.474	95
	0.100	0.083	83	0.100	0.116	116	0.100	0.065	65	0.100	0.087	87	0.100	0.071	71
Cyfluthrin	1.60	1.41	88	1.60	1.34	84	1.60	1.49	93	1.60	1.49	93	1.60	1.54	96
	0.160	0.170	106	0.160	0.157	98	0.160	0.134	84	0.160	0.156	97	0.160	0.136	85
	0.032	0.033	103	0.032	0.034	106	0.032	0.029	92	0.032	0.027	84	0.032	0.021	65
Flucythrinate	3.00	2.69	90	3.00	2.39	80	3.00	2.73	91	3.00	1.78	59	3.00	2.97	99
	0.300	0.367	102	0.300	0.296	99	0.300	0.261	87	0.300	0.211	70	0.300	0.235	78
	0.060	0.060	100	0.060	0.056	93	0.060	0.051	85	0.060	0.044	73	0.060	0.038	63
Fenvalerate	2.00	1.81	91	2.00	1.70	85	2.00	1.81	91	2.00	1.98	99	2.00	1.87	94
	0.200	0.215	107	0.200	0.203	102	0.200	0.178	89	0.200	0.201	100	0.200	0.156	78
	0.040	0.041	103	0.040	0.042	106	0.040	0.035	87	0.040	0.033	82	0.040	0.024	60
Fluvalinate	2.00	1.97	99	2.00	1.65	83	2.00	1.68	84	2.00	1.72	86	2.00	1.88	94
	0.200	0.210	105	0.200	0.192	96	0.200	0.168	84	0.200	0.199	99	0.200	0.179	90
	0.040	0.041	103	0.040	0.041	103	0.040	0.033	83	0.040	0.031	78	0.040	0.026	65
Deltamethrin	2.00	1.82	91	2.00	1.72	86	2.00	1.78	89	2.00	2.43	121	2.00	1.87	94
	0.200	0.214	107	0.200	0.196	98	0.200	0.174	87	0.200	0.242	121	0.200	0.206	103
	0.040	0.041	103	0.040	0.039	98	0.040	0.035	87	0.040	0.033	83	0.040	0.029	72

[a] Besides the 5 crops listed in this table, recovery data for potatoes and bananas also are satisfactory.

Table 5. Precision data from maize and apple samples (n = 10)

	Maize				Apple			
Pesticide	Added, ppm	Av. rec., %	SD, %	CV, %	Added, ppm	Av. rec., %	SD, %	CV, %
py-115	0.10	102.9	8.1	7.9	0.010	103.9	12.6	12.1
Allethrin	0.10	94.2	5.9	6.3	0.010	62.3	5.8	9.2
Biphenthrin	0.14	89.5	5.6	6.3	0.014	84.2	3.9	4.7
Cyhalothrin	0.10	88.7	5.0	5.6	0.010	80.3	3.3	4.1
Permethrin	0.50	82.0	5.3	6.5	0.050	87.3	4.9	5.7
Cyfluthrin	0.16	81.9	5.7	7.0	0.016	83.4	4.6	5.5
Flucythrinate	0.30	83.8	5.9	7.0	0.030	72.7	4.1	5.7
Fenvalerate	0.20	81.8	5.1	6.2	0.020	82.0	3.4	4.1
Fluvalinate	0.20	77.4	11.3	14.6	0.020	78.9	4.7	5.9
Deltamethrin	0.20	84.8	4.7	5.5	0.020	82.8	3.5	4.2

and Shanghai Midwest Pesticide Factory for providing reference pesticides.

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RESIDUES AND TRACE ELEMENTS

Spectrophotometric Determination of Decamethrin and Its Residues in Insecticidal Formulations and in Water

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Three spectrophotometric methods were developed for the microdetermination of decamethrin in insecticidal formulations and in water. The methods are based on the hydrolysis of decamethrin with methanolic KOH to 3-phenoxybenzaldehyde; condensation of the hydrolysis product with 2,4-dinitrophenylhydrazine (2,4-DNPH), 4-nitrophenylhydrazine (4-NPH), or 2,4,6-trinitrophenylhydrazine (2,4,6-TNPH) under alkaline conditions; and measurement of the condensates at the absorption maxima of 444, 535, and 480 nm, respectively. The relationship between absorbance and concentration was linear in the ranges of 0.1–5.0 μg/mL, 0.5–7.0 μg/mL and 0.1–5.5 μg/mL for 2,4-DNPH, 4-NPH, and 2,4,6-TNPH, respectively. The methods are sufficiently sensitive and can be used to detect decamethrin at concentrations as low as 0.1 μg/mL.

Decamethrin, (*S*)-α-cyano-3-phenoxybenzyl-(1*R*, 3*R*)-3-(2,2-dibromovinyl)-2,2-dimethylcyclopropan-1-carboxylate, is a broad spectrum insecticide with nonsystemic activity. It is a very powerful pyrethroid insecticide that acts both by direct contact and by ingestion. It is extremely stable on exposure to air, but when exposed to UV irradiation or sunlight, it undergoes cis–trans isomerization, splitting of the ester bond, and loss of bromine. It is more stable in acidic media than in alkaline media. Decamethrin has been very widely applied (1, 2). It is a fast-acting insecticide for controlling lepidoptera, homoptera, and coleoptera in a wide range of field crops, fruits, vegetables, ornamentals, and flowers. It is used in the control of flying and crawling insects in households, animal houses, and stored products. It has also been used extensively for residual indoor applications against mosquitoes, bedbugs, cockroaches, and flies in India and many tropical countries. It has applications as a wood preservative and as an animal ectoparasiticide.

Decamethrin is not phytotoxic but is toxic to bees, fish (LC_{50} is 0.001–0.01 mg/L), birds (acute oral LD_{50} for mallard ducks is >5000 mg/kg), and mammals (acute oral LD_{50} for male and female rats is 128 and 139 mg/kg, respectively, in vegetable oil and for both male and female rats is >5000 mg/kg in aqueous solution.) The acute percutaneous LD_{50} for rats and rabbits is >2000 mg/kg.

After application, decamethrin may enter lakes, streams, and ponds due to runoff water from treated areas. Thus, a rapid, sensitive, and reliable method for the determination of its residues has become imperative. Decamethrin residues in grain, vegetables, treated oils, fruits, plants, crops, water, air, soil, insecticidal formulations, fat, brain tissues of treated rats, spiked urine, feces, milk, butter, liver, kidneys, muscle, animal tissue, flour, tea, and fish eggs have been analyzed by gas chromatographic (3–14), gas-liquid chromatographic (15–17), liquid chromatographic (18–24), and thin-layer chromatographic (25) methods. But their use is often limited due to high cost (i.e., the equipment is unavailable in all laboratories). This restriction necessitated a search for other methods. In this paper, we report on new, simple, lower-cost, and accurate spectrophotometric methods.

Experimental

Apparatus

(a) *Spectrophotometer.*—Shimadzu Model UV-240, with 1 cm quartz cell and recorder.

(b) *pH meter.*—Elico Model LI-120A digital, with combined glass electrode.

Reagents and Materials

(a) *Decamethrin (deltamethrin).*—2.8% Emulsifiable formulation (Evid and Co. Chem, Bharuch, Gujarat, India), and standard, 96% purity (Hoechst (I) Ltd., Bombay, India); working standard solution containing 100 μg/mL decamethrin in methanol was used.

(b) *Solvents.*—All organic solvents (e.g., methanol, chloroform, methylene chloride) were acetone-free, analytical grade, and distilled in glass (s.d. fine - Chem, Bombay, India).

(c) *2,4-Dinitrophenylhydrazine (2,4-DNPH).*—(Aldrich Chemical Co., Inc., Milwaukee, WI 53201); 0.1% solution in methanol containing 0.5 mL concentrated HCl was used.

(d) *4-Nitrophenylhydrazine (4-NPH).*—Analytical reagent grade (s.d. fine - Chem, Bombay, India); 0.1% solution in methanol containing 0.5 mL concentrated HCl was used.

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(e) *2,4,6-Trinitrophenylhydrazine (2,4,6-TNPH).*—Synthesized according to Rappoport and Sheradsky (26); 0.1% solution in methanol containing 0.5 mL concentrated HCl was used.

(f) *Potassium hydroxide (KOH).*—Analytical reagent grade (s.d. fine - Chem, Bombay, India); 2 and 4% solutions in methanol were used.

(g) *Hydrochloric acid (HCl).*—35.4%, sp gr 1.18. (s.d. fine - Chem, Bombay, India); 0.2N in distilled water.

(h) *Sodium sulfate (Na_2SO_4).*—Anhydrous, analytical reagent grade (British Drug Houses, A Division of Glaxo Laboratories (I) Ltd., Bombay, India).

Preparation of Standard Curve

Aliquots containing 0.00, 0.01, 0.05, 0.1, 0.2, 0.4, 0.6, and 0.8 mL decamethrin working standard solution (1–80 µg) were placed in clean, dry, 10 mL graduated test tubes. One milliliter 2% methanolic KOH was added to each test tube, mixed well by shaking, and heated on a water bath at 45°C for 30 min. The test tubes were then removed from the water bath and cooled to room temperature for 10 min. The excess alkali was neutralized with 0.2 N HCl. One milliliter 2,4-DNPH solution in methanol was added to each test tube, followed by 1 drop of concentrated HCl. The contents were thoroughly mixed by shaking, heated on a water bath at 55°C for 30 min, cooled, and added to 2 mL 4% methanolic KOH solution. An intense red-colored solution was obtained, due to the formation of a quinonoid compound. The absorbance of the resulting solution was measured at 444 nm with a spectrophotometer against a blank. The plot of decamethrin concentration (0.1–5.0 µg/mL) vs absorbance yielded a straight line.

The procedure described above was also followed in preparing standard curves for the determination of decamethrin using the other 2 reagents, 4-NPH and 2,4,6-TNPH. For each determination, 1.5 mL 4-NPH or 2.5 mL 2,4,6-TNPH was added, followed by 5.5 mL or 2.0 mL methanolic KOH, respectively. A reddish-purple solution with an absorption maxima at 535 nm (for 4-NPH) or a red solution with an absorption maxima at 480 nm (for 2,4,6-TNPH) was obtained. The absorbances of the resulting solutions were measured with a spectrophotometer (Figure 1). Plots of decamethrin concentration (0.5–7.0 µg/mL for 4-NPH and 0.1–5.5 µg/mL for 2,4,6-TNPH) vs absorbance yielded straight lines.

Determination of Decamethrin

(a) *Formulations.*—0.9 mL (25 mg) decamethrin insecticidal formulations was placed in a porcelain dish and 10 mL methanol was added. This mixture was stirred well, and then the solvent was evaporated by heating the samples on a hot water bath. The procedure was repeated 5 times, and the resulting solution was diluted to 25 mL with methanol in a calibrated flask. Known amounts of this solution were used for color development, in accordance with the procedure outlined under standard curve preparation.

(b) *Water (distilled and cattle-dip).*—*(1)* Distilled water samples (1 L) and *(2)* cattle-dip water samples (1 L) were collected, filtered through Whatman No. 42 filter paper, and fortified with 25 mg pesticide in 5 mL methanol. The pH of the samples was adjusted to between 3 and 4 with 50% sulfuric acid, and 10 g anhydrous Na_2SO_4 was dissolved in each sample. The pyrethroid in the samples was extracted 3 times by using 50 mL chloroform for each extraction and by shaking for ca 10 min. The extracts were combined and washed with 10 mL 0.1M K_2CO_3 solution to break up any emulsions. The chloroform solution was dried over 10 g anhydrous Na_2SO_4, and the solvent then evaporated by exposure to air. The remaining pesticide residue was dissolved in methanol and diluted to 250 mL in a calibrated flask. Known amounts of this solution were used for color development, as described under *Preparation of Standard Curve.*

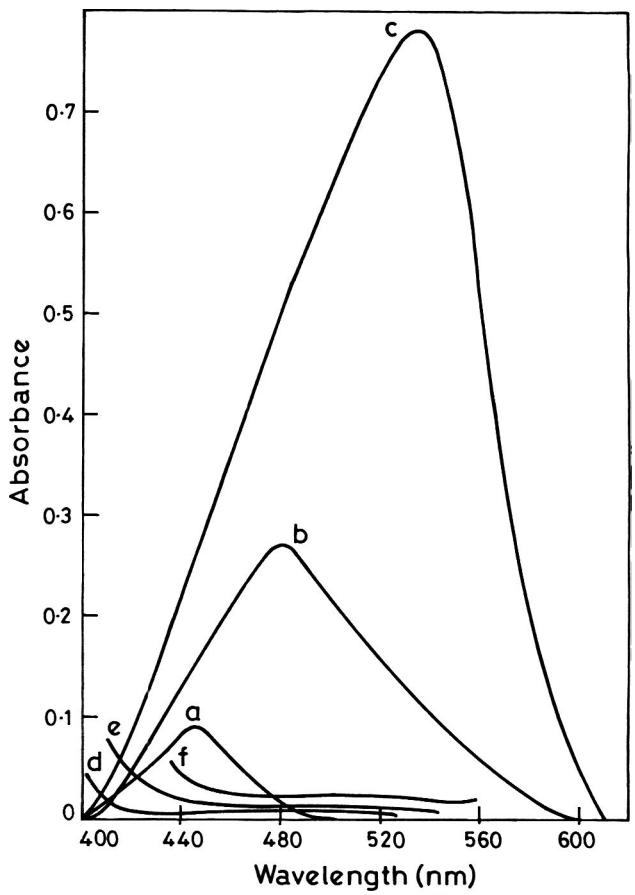

Figure 1. Absorption spectra of (a) Decamethrin-2,4-DNPH; (b) Decamethrin-2,4,6-TNPH; (c) Decamethrin-4-NPH; (d) 2,4-DNPH vs blank; (e) 2,4,6-TNPH vs blank; and (f) 4-NPH vs blank.

Interference from Aldehydes

The water samples (1 L each) were fortified with known amounts of pesticide dissolved in 5 mL methanol. Known amounts of benzaldehyde dissolved in 10 mL methanol were added, and the pH of each solution was adjusted to between 3 and 4 with 50% sulfuric acid. Ten grams Na_2SO_4 was dissolved in each sample, and the pesticide along with the aldehyde was extracted 3 times, using 50 mL chloroform for each extraction. The extracts were combined and placed in a 500 mL round-bot-

Table 1. Optical characteristics, precision, and accuracy of 3 spectrophotometric methods for the microdetermination of decamethrin

Pesticide	Reagent	Maximum color development, h	pH range	Stability period, h	Molar absorptivity $\times 10^4$, $mol^{-1}cm^{-1}$	Sandell's sensitivity, $\mu g/cm^2$	RSD, %	CV, %, p = 0.05
Decamethrin	2,4-DNPH	Immediate	11.0–12.0	46	5.55	0.009	0.23	± C.0012
	4-NPH	Immediate	11.0–12.5	50	4.95	0.010	0.25	± C.0012
	2,4,6-TNPH	Immediate	11.0–12.5	40	7.71	0.007	0.35	± C.0019

tom flask, into which 100 mg metachloroperbenzoic acid was dissolved. The resulting solution was refluxed on a hot water bath for ca 15 min to convert the aldehyde into an acid. Thereafter, the solution was cooled, washed 3 times with 25 mL 0.2M sodium bicarbonate solution per wash to remove the acid and unreacted metachloroperbenzoic acid, and finally washed 3 to 4 times with distilled water, using 50 mL for each washing, to remove the excess carbonate. The chloroform solution was then dried over 10 g anhydrous Na_2SO_4, and the solvent was evaporated by exposure to air. The residue obtained was dissolved in methanol and then diluted to 250 mL with methanol in a calibrated flask. Known amounts of this solution (ranging from 0.1 to 0.5 mL) were placed in 10 mL graduated test tubes. The determination of decamethrin was carried out with the reagents 2,4-DNPH, 4-NPH, and 2,4,6-TNPH, as described under *Preparation of Standard Curve.*

The procedures described above were also carried out employing methylene chloride in place of chloroform as the solvent.

Results and Discussion

The time required for maximum color development, stabilities, molar absorptivities, Sandell's sensitivities, relative standard deviations, and confidence limits of the colored products for the 3 methods are given in Table 1. Results of decamethrin determination in insecticidal formulations and water samples are shown in Table 2. The data on decamethrin determination in water samples containing an aldehyde are presented in Table 3.

Decamethrin on alkaline hydrolysis gives 3-(2.2-dibromovinyl)-2,2-dimethylcyclopropan-1-carboxylic acid and 3-phenoxybenzaldehyde. This aldehyde, upon condensation with 2,4-DNPH, yields the corresponding phenylhydrazone. The hydrazone formed in the presence of KOH changes to quinonoid form (27), which is a red-colored compound with wavelength of absorption maxima (λ_{max}) at 444 nm. The course of reactions with 4-NPH and 2,4,6-TNPH are similar, and the final quinonoid products possess reddish-purple

Table 2. Determination of decamethrin in insecticidal formulations containing 2.8% decamethrin and in water samples

Reagent	Decamethrin, 2.8%			Decamethrin added, µg	Distilled water		Cattle-dip water	
	Sampled, µg	Found, µg	Formulation, %		Found, µg[a]	Rec., %	Found, µg[a]	Rec., %
2,4-DNPH	1.0	0.95	2.66	1.0	0.93 ± 0.12	93.0	0.95 ± 0.15	95.0
	2.0	1.96	2.74	2.0	1.96 ± 0.15	98.0	1.96 ± 0.13	98.0
	3.0	2.93	2.73	3.0	2.96 ± 0.30	98.7	2.93 ± 0.28	97.7
	4.0	3.96	2.77	4.0	3.96 ± 0.21	99.0	3.96 ± 0.24	99.0
	5.0	4.98	2.78	5.0	4.98 ± 0.31	99.6	4.95 ± 0.30	99.0
		AV. 2.73						
4-NPH	1.0	0.95	2.66	1.0	0.93 ± 0.12	93.0	0.93 ± 0.37	93.0
	2.0	1.90	2.66	2.0	1.87 ± 0.13	93.5	1.88 ± 0.38	94.0
	3.0	2.90	2.71	3.0	2.90 ± 0.20	96.7	2.90 ± 0.41	96.7
	4.0	3.95	2.76	4.0	3.95 ± 0.15	98.8	3.95 ± 0.32	98.8
	5.0	4.90	2.74	5.0	4.90 ± 0.18	98.0	4.90 ± 0.56	98.0
		AV. 2.71						
2,4,6-TNPH	1.0	0.95	2.66	1.0	0.93 ± 0.30	93.0	0.93 ± 0.21	93.0
	2.0	1.90	2.66	2.0	1.88 ± 0.31	94.0	1.90 ± 0.30	95.0
	3.0	2.90	2.71	3.0	2.90 ± 0.29	96.7	2.85 ± 0.32	95.0
	4.0	3.90	2.73	4.0	3.85 ± 0.32	96.3	3.85 ± 0.40	96.3
	5.0	4.90	2.74	5.0	4.95 ± 0.42	99.0	4.95 ± 0.42	99.0
		AV. 2.70						

[a] Average ± standard deviation of 5 analyses.

Table 3. Interference from benzaldehyde in water samples

Decamethrin added, μg	Benzaldehyde added, μg	Decamethrin	
		Found, μg	Rec., %
1	40	0.96	96.0
2	80	1.96	98.0
3	120	2.93	97.7
4	160	3.96	99.0
5	200	4.95	99.0

(λ_{max} = 535 nm) and red colors (λ_{max} = 480 nm), respectively. The determination of decamethrin in insecticidal formulation emulsion shows that the average decamethrin content obtained with these methods varied from 2.70 to 2.73%, compared with the declared value of 2.8%. To check the recovery of decamethrin by these methods, water samples were spiked with known amounts of decamethrin and analyzed. Recoveries of decamethrin from spiked water samples (distilled and cattle-dip) varied from 93.0 to 99.6%. Determination of decamethrin from water samples containing an aldehyde gave recovery values ranging from 96 to 99%.

The 3 proposed methods are simple, rapid, sensitive, and selective and can be used for the determination of decamethrin in trace amounts (≤0.1 μg). Interference from many substances other than aldehydes is eliminated by the selective extraction procedure used and also by measuring the absorbance of the sample against that of a corresponding crop control (blank). Additional advantages of these methods are that colors develop instantaneously and are stable for long periods of time (Table 1). Beer's law is obeyed in the ranges 0.1–5.0 μg/mL for 2,4-DNPH, 0.5–7.0 μg/mL for 4-NPH, and 0.1–5.5 μg/mL for 2,4,6-TNPH. The reagents have minimal absorptions at the wavelengths of maximal absorption of the colored compounds. Thus, excess reagent has no effect on the absorbance of the colored compound. Moreover, these methods do not involve the elaborate cleanup procedures required by other methods and can be suitably adopted for routine checkup of the purity of decamethrin formulations and the determination of decamethrin in polluted environmental samples. Notably, the procedure developed for the determination of decamethrin from water samples containing an aldehyde is simple and quite satisfactory. Finally, the determination of decamethrin can also be carried out using methylene chloride instead of chloroform.

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RESIDUES AND TRACE ELEMENTS

Liquid Chromatographic Determination of Norflurazon and Its Initial Metabolite in Soil

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A rapid, sensitive method for the determination of norflurazon in 4 soils is described. Data on the initial soil metabolite is also obtained in soils with low organic matter. The method consists of extraction of soil samples with methanol, filtration, liquid chromatographic separation of methanol-soluble components by using a C_{18} column, and fluorescence detection with excitation at 294 nm and emission measured at 398 nm. Recoveries from fortified soils were >90% for norflurazon and >80% for desmethylnorflurazon from the Shipps, Lexington, and Harkey soils. Average percent relative standard deviations over the soils examined was 5.5% for norflurazon and 8.7% for desmethylnorflurazon. The limit of detection for norflurazon was 10 ng/g soil, whereas the limit of detection for desmethylnorflurazon was 100 ng/g soil because of its smaller relative detector response.

Norflurazon [4-chloro-5(methylamino)-2-(3-(trifluoromethyl)phenyl)-3(2*H*)-pyridazinone] is a soil-applied herbicide registered for use in cotton, soybeans, citrus, and other crops (Figure 1). The typical application rate is from 1 to 2 kg/ha. Norflurazon has a water solubility of 28 µg/mL and a vapor pressure of 2.0×10^{-8} mm Hg at 20°C (1). Norflurazon's initial soil metabolite, desmethylnorflurazon (DMN), has been identified in field dissipation studies (2).

The literature contains several methods using gas chromatography (GC) with an electron capture detector for the determination of norflurazon in soil (2–4). Schroeder (2) extracted several Georgia soils (organic matter, 0.7–2.8%) with methanol or acetonitrile, with norflurazon recoveries ranging from 93 to 102%. Methods by Winkler (3) and a method supplied by the manufacturer of this crop protection chemical (4) require extraction and cleanup techniques followed by GC analysis. Limits of detection of the GC methods were approximately 10 ng/g soil. Draper and Street (5) reported an isocratic liquid chromatographic method using UV detection of norflurazon and desmethylnorflurazon in fruit crops. The method involved liquid–liquid partitioning and other cleanup procedures. The minimum detectable quantity for each residue was <0.1 µg/g in the crops tested.

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Analytical methodology was developed for the simultaneous determination of both norflurazon and its initial soil metabolite at ppm to ppb levels in soil, using an external standard liquid chromatographic (LC) method with fluorescence detection. The methodology could be used to support studies on the bioavailability and environmental fate of norflurazon.

Experimental

Apparatus and Reagents

(a) *LC system.*—Waters liquid chromatograph, including Model 680 control unit, Model 715 autoinjector, Model 501 solvent delivery system, Model 470 fluorescence detector set to excite at 294 nm and read emission at 398 nm, 4.0 s filter, 1000× gain, and ATTN = S (Milford, MA). Additionally, a Hewlett-Packard (San Fernando, CA) Model 3396 integrator was used to interpret detector response.

(b) *Analytical column.*—25 cm × 4.6 mm id, 5 µm, LC-C_{18}; in-line 1 cm × 1.5 mm pellicular C_{18} guard column (Alltech, Chicago, IL).

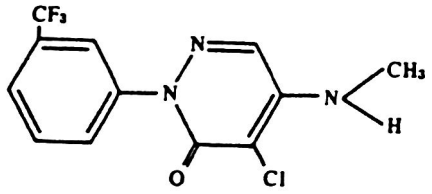
(c) *Solvents.*—LC grade (J.T. Baker Inc., Phillipsburg, NJ, and Burdick and Jackson, Muskegon, MI).

(d) *Mobile phase.*—Initially acetonitrile–water (50 + 50); 0–12 min linear gradient to 75 + 25 (v/v).

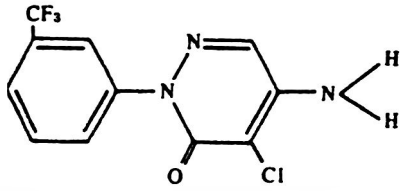
(e) *Analytical standards.*—Norflurazon and desmethylnorflurazon (Sandoz Crop Protection, Des Plaines, IL). Standards were >99% pure, and each was used without purification.

Soil Selection

Soil was selected to represent a range in texture, organic matter content, and pH (Table 1). Soil 1 is a Shipps clay (high clay content); soil 2 is a Lexington silt loam; soil 3 is a Harkey loam (high pH); and soil 4 is a Drummer silty clay loam (high organic matter content). All soils were passed through a 10-mesh (2 mm) sieve prior to analysis. Soils 1, 2, and 3 represent soils upon which norflurazon is typically used. Soil 4 would not represent a soil upon which norflurazon is routinely applied but was included to determine the effect of relatively high organic matter content on norflurazon quantitation.



NOR



DMN

Figure 1. Norflurazon (NOR) and its initial soil metabolite desmethylnorflurazon (DMN).

Fluorescence Detection

The fluorescence properties of norflurazon when dissolved in acetonitrile were examined previously (6). Fluorescence was maximum when excitation was at 294 and emission measured at 398 nm. Adding water or acidifying the solution had no effect on the observed fluorescence. The relative fluorescence was approximately one-fourth that of fluometuron (6), for which a similar method was reported (7).

Several acetonitrile–water combinations were evaluated for herbicide and metabolite retention time and capacity factor (k') (8). The accuracy of the analysis was examined by injection of a series of standards containing norflurazon and desmethylnorflurazon in methanol at 0.05–5 μg/mL. Injection volume was 50 μL. This range corresponded to herbicide soil concentrations of 0.10–10 μg/g by the described methodology.

Extraction

Analytical norflurazon and desmethylnorflurazon (in methanol) was added to duplicate 250 mL polyethylene bottles with screw-top caps (Nalgene, Rochester, NY) containing 40 g air-dried soil to achieve soil concentrations of 940 and 830 ng/g, respectively. The methanol was allowed to evaporate, bottles were capped, and the contents were mixed thoroughly by hand shaking. The bottles were then allowed to equilibrate statically for 24 h. Additional treatments included norflurazon and desmethylnorflurazon added to bottles containing no soil and each soil without norflurazon or desmethylnorflurazon. The 4 soils were examined for extraction efficiency in a single experiment. Methanol (80 mL) was added to each bottle before they were placed on a shaker (Eberbach, Ann Arbor, MI) operated at 180 rpm for 6 h. The extract was filtered through 2 Whatman No. 1 filter papers (Whatman, Clinton, NJ) directly into 4 mL autosampler vials.

Results and Discussion

Injection of 50 μL of norflurazon and desmethylnorflurazon standards produced a peak with a retention time of 3.4–19.3 min, depending on mobile-phase composition (Table 2). At equal relative concentrations, methanol-containing mobile phases had greater capacity factors than those containing acetonitrile. Chromatographic concerns associated with the isocratic procedures for both methanol and acetonitrile mobile phases included poor resolution of norflurazon and desmethylnorflurazon (capacity factor differences, <0.5) and asymmetrical peak shape (peak tailing). The use of methanol also was coincident with higher system operation pressures, although the pressure could be reduced by the use of a column oven. To overcome these problems, gradient elution using acetonitrile–water (gradient 2) was used for subsequent quantitation (Figure 2). The norflurazon standard curve was linear in the 50–2000 ng/mL range, with an r^2 of 0.99 (n = 7, data not shown) but was nonlinear at higher concentrations (>2000 ng/mL). With the proposed methodology, concentrations in soil extracts above this limit would not be expected from typical soil applications of norflurazon. The norflurazon analytical standards (in methanol) and methanol soil extracts were stable over time and showed no change in detector response when stored at room temperature for 8 weeks. Soil extracts of desmethylnorflurazon were not stable, and detector response decreased by approximately 15% after 2 weeks.

Norflurazon and desmethylnorflurazon recoveries from all soils ranged from 81 to 108% (Table 3). Desmethylnorflurazon was not determined in soil 4 because of background interference. The precision of the method was good, as indicated by an average relative standard deviation (RSD) of 6.8% for all recoveries (Table 3).

The standard curve for desmethylnorflurazon was linear from 0 to 5000 ng/mL, with r^2 = 0.99 (n = 8, data not shown). The detector response from this initial metabolite was 10 times smaller than the parent compound. Fresh solutions were prepared 3 times to make sure the lower detector response was not a laboratory error. Each preparation of stock solutions and sub-

Table 1. Soil characteristics

Soil	Series	State	Sand, %	Silt, %	Clay, %	Organic matter, %[a]	pH[b]
1	Shipps	Texas	1	32	67	1.4	7.3
2	Lexington	Tennessee	4	75	21	1.2	6.4
3	Harkey	New Mexico	36	39	25	1.1	7.2
4	Drummer	Illinois	14	53	33	4.8	6.3

[a] Determined by modified Debolt version of Walkley–Black method.
[b] Determined by 1:2 soil:water suspension technique.

Table 2. Solvent systems and retention times for norflurazon (NOR) and desmethylnorflurazon (DMN)

Mobile phase	Solvent ratio (v/v)	Retention time, min		k'^a	
		DMN	NOR	DMN	NOR
Acetonitrile–water	80 + 20	3.45	3.79	0.35	0.49
Acetonitrile–water	70 + 30	4.00	4.58	0.57	0.80
Acetonitrile–water	60 + 40	4.92	6.11	0.93	1.40
Acetonitrile–water	50 + 50	7.03	9.55	1.76	2.75
Acetonitrile–water	40 + 60	12.83	19.29	4.03	6.56
MeOH–water	80 + 20	4.36	4.88	0.71	0.91
MeOH–water	70 + 30	7.12	8.31	1.79	2.26
Acetonitrile–water	Gradient 1[b]	8.40	12.65	2.29	3.96
Acetonitrile–water	Gradient 2[c]	7.43	10.43	1.91	3.09

[a] Capacity factors for norflurazon and desmethylnorflurazon; t_o for system = 2.55 min.
[b] Acetonitrile–water (v/v), initial conditions of (45 + 55), 0 to 12 min linear gradient to (60 + 40).
[c] Acetonitrile–water (v/v), initial conditions of (50 + 50), 0 to 12 min linear gradient to (75 + 25).

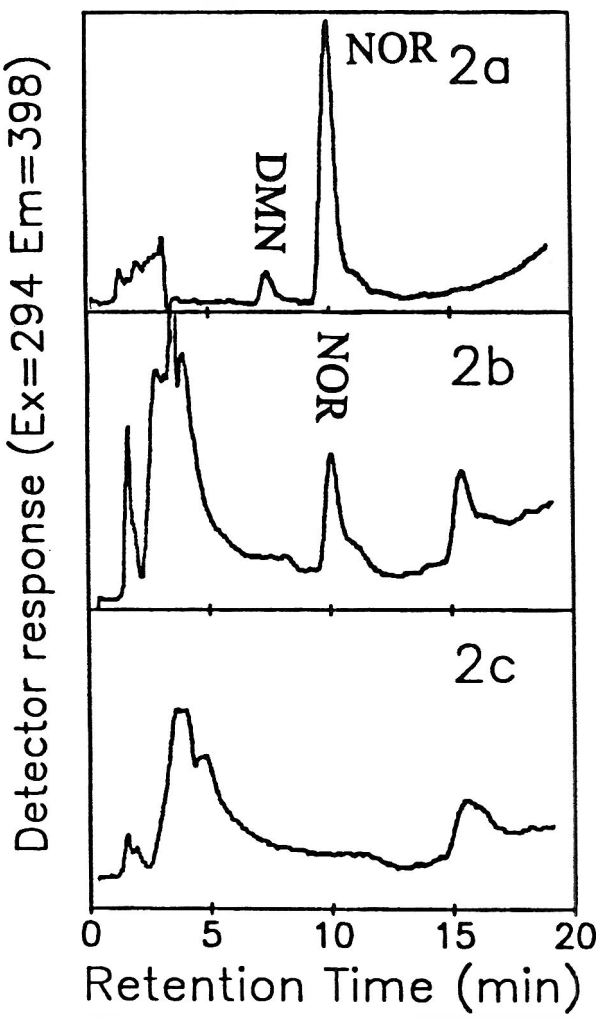


Figure 2. Liquid chromatograms of (a) analytical standard containing desmethylnorflurazon (DMN) at 1000 ng/g and norflurazon (NOR) at 1000 ng/g, (b) soil 1 extract containing norflurazon at 500 ng/g, and (c) soil 1 extract containing no norflurazon or desmethylnorflurazon.

sequent analysis produced the same result. Desmethylnorflurazon possesses the same rings as the parent, but apparently, the loss of the methyl group reduces fluorescence at the measured parameters because of changes in the electron distribution around the ring. This smaller detector response limits the utility of this method to quantitate trace amounts of desmethylnorflurazon. Desmethylnorflurazon was not discernable in extracts from soil 4. This soil has a large organic matter content (for a mineral soil), which interfered with the small desmethylnorflurazon detector response. Norflurazon is not routinely used in soils with organic matter content of >4%, and we realize and acknowledge this limitation in the method to quantitate desmethylnorflurazon in this soil. This limitation could be addressed by cleanup procedures or other techniques.

Baseline interferences with the proposed methodology were minimal when compared with UV detection at 275 nm (data not shown). Other commonly used herbicides are not expected to interfere with norflurazon quantitation, because many do not fluoresce under these test conditions (6), with the exception of fluometuron. A previous paper has discussed fluometuron analysis (7). Chromatographic separation of fluometuron and norflurazon is possible, but the metabolites of each compound coelute with the other compounds of interest. This makes simultaneous determinations of the 2 herbicides difficult.

With a signal-to-noise ratio of 2, a conservative limit of quantitation for this methodology is 10 ng/g soil for norflurazon. Because of the weaker detector response, the limit of detection of desmethylnorflurazon is 100 ng/g soil. The simple extraction method presented here combined with gradient elution and fluorescence detection would allow the rapid determination of these compounds in large numbers of soil samples at levels suitable for many purposes. This method has been used for the analysis of >400 soil samples from surface and subsurface soil samples, with the presented chromatograms being from actual field samples (Figure 2). For trace analysis of these compounds, or the analysis of these compounds in matrixes containing substantial amounts of organic material, modifications of the extraction and chromatographic procedures may be

Table 3. Recovery of norflurazon and desmethylnorflurazon from 4 soils with methanol extraction, gradient elution, and fluorescence detection[a]

Soil	Series	Parent or metabolite	Detected concn, ng/g soil	Rec., %	RSD, %
1	Shipps	Norflurazon	860	92	7.0
		Desmethylnorflurazon	670	81	8.7
2	Lexington	Norflurazon	960	102	4.9
		Desmethylnorflurazon	900	108	17.2
3	Harkey	Norflurazon	930	99	1.2
		Desmethylnorflurazon	750	90	0.1
4	Drummer	Norflurazon	870	93	8.8
		Desmethylnorflurazon[b]	—	—	—

[a] Initial concentrations: norflurazon, 940; desmethylnorflurazon, 830.
[b] Not determined because of background interference.

necessary. The proposed methodology should serve as a good foundation for method development and refinement, and the sensitivity and selectivity provided by fluorescence detection should provide a sound basis for chemical analysis of these compounds.

Acknowledgments

We thank Morris Merkle (Texas), Jill Schroeder (New Mexico), and Robert Hayes (Tennessee) for providing soil to perform the studies.

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TECHNICAL COMMUNICATIONS

Quantitative Procedure for Chlorophyllin Copper Complex

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The reliability of the currently used quantitative assay for commercially available chlorophyllin copper complex is discussed. It was shown that optical measurements at 405 nm can overestimate the purity of the preparation by 16.4–49.5%. This conclusion is from comparative studies using spectrophotometry at 405 nm and elemental analysis (copper content) of chlorophyllin copper complex. Spectrophotometry at 630 nm resulted in closer agreement with data calculated from elemental analysis. These observations have to be taken into consideration by those involved in the manufacture and distribution of chlorophyllin copper complex as well as in the development of the United States Pharmacopeia compendial monograph for this preparation.

A few decades ago, a group of plant porphyrin-based compounds, chlorophyll and its derivatives, were found to exhibit some useful technical and pharmaceutical properties. Lately, the most notable among these preparations, chlorophyllin copper complex (CCC), was successfully introduced to the U.S. market as a coloring agent, cosmetic ingredient, health food additive, deodorant drug for internal use, and wound healing remedy (1, 2). In the past 10 years, CCC was listed by more than 60 U.S. companies involved in distribution of CCC and/or end products with CCC included (3–5).

CCC is obtained from chlorophyll under alkaline treatment followed by the introduction of copper. Depending upon the raw material and technological protocol used, saponification of chlorophyll can lead to hydrolysis of the phytyl and methyl ester groups, cleavage of the cyclopentanone ring, and other secondary reactions. Although it is generally accepted that chlorophyll derivatives in CCC are coordinated with copper, some may be analogues lacking metal. Chromatographic studies indicate that CCC can contain up to a dozen of chlorophyll-based constituents. Only 2 have been identified as major components (Figure 1) (6).

Although CCC has a long history of commercialization in the United States, there is no U.S. Food and Drug Administration (FDA)-approved analytical method to monitor the quality of this preparation. In this study, different methodologies for the quantitative evaluation of commercially available CCC were compared by using data from elsewhere (7) and this laboratory.

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METHODS

Optical and elemental (copper content) analyses were conducted. In the currently used spectrophotometric assay, designated as the NNR method (8), readings of CCC absorption have been performed in phosphate buffer of pH 7.5 at the Soret band (405 nm). Another spectrophotometric procedure, described by Wall (9), was also used. Measurements of CCC were conducted in a 50% aqueous ethanol solution at the red absorption maximum (630 nm). The amount of CCC estimated on the basis of its copper content was determined in accordance with our theoretical calculations. It was established that 100% pure, coppered CCC contains an average of 9.2% chelated copper as determined for sodium copper chlorin e_6 and sodium copper isochlorin e_4, the main chlorophyll derivatives identified (Figure 1). The data collected from spectrophotometry and elemental analyses were grouped corresponding to the source of production of the CCC.

Results and Discussion

Quantitation of CCC by the NNR method gives results that do not conform with those obtained by other procedures. The data presented in Table 1 illustrate clearly this discrepancy. The purity of CCC in different batches, as determined by this method, was 78.8–99.5% for the U.S. preparations and 39.2–53.5% for those produced elsewhere. The copper contents of these 2 groups of samples were between 4.1–4.6 and 2.1–3.3%, respectively. These values correspond to purities of only 44.6–50.0% and 22.8–35.9%, based on the amount of copper in CCC manufactured in the United States and elsewhere, respectively. That is, the purity of CCC, as established by copper content, differs from the values obtained by the NNR measurements by 34.2–49.5% and 16.4–17.6%, respectively, for the groups of the samples analyzed.

The NNR assay in its present version, therefore, can overestimate the purity of CCC by 1.5–2.0 times. This difference may vary as more analytical data on CCC are collected. By contrast, the spectrophotometric measurements of the amount of CCC at the red absorption maximum (630 nm) gave better agreement with those calculated on the basis of copper content.

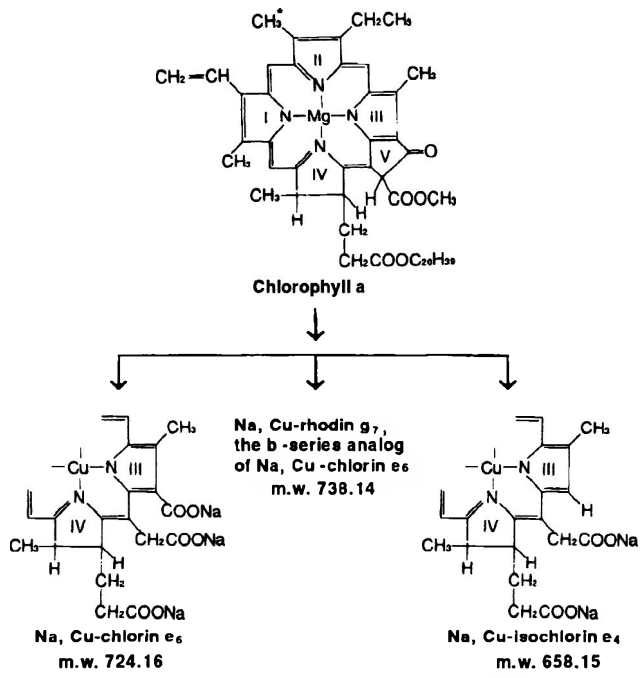

Figure 1. Structure of chlorophyll a and major related derivatives identified in CCC (6).

The purity of CCC determined by this assay was 26.5–34.2%, and the copper content estimation of purity was 22.8–35.9%.

The data shown in Table 1 indicate that CCC manufactured elsewhere is of lower quality compared with CCC produced in the United States. The purity of the former is approximately 50–70% of the latter. These results point to the variability of the chemical composition in CCC that may come about due to differences in production methods.

The lack of a reliable analytical procedure for CCC has had a negative impact on the selection of its appropriate specification values. The typical range of 4–6% for the copper content in CCC was given by the Cosmetic, Toiletry, and Fragrance Association (10) and was adopted by FDA (11). On the basis of the theoretical calculation mentioned above, these values correspond to a preparation with purity of only 43.5–65.2%. This value conflicts with the CCC results given in ref. 10 as 95–105% and as a "total color, not less than 75%" (11) established for a "good manufacturing practice." The chlorophyll-based compounds in CCC can be described in general terms as macrocyclic tetrapyrrols, but the structures of contaminating substances, which may account for up to 50% and more of this preparation, are completely unknown. These impurities may be various plant organic compounds, resulting in the CCC composition being entirely dependent upon the raw material used. Algae, silk worm excreta, alfalfa, pine needles, and plant by-products from the agricultural and pharmaceutical industries are used to obtain CCC. The variance in composition of nonporphyrin compounds as well as the possible presence of non-coppered chlorophyll derivatives with photosensitizing activity, in turn, can alter the biological properties of CCC. Furthermore, because of the variance in chemical and biological properties, certain side effects from the use of CCC may be expected (12).

Table 1. Estimation of CCC purity by spectrophotometry and elemental analysis

Source of CCC	Purity, %		
	Spectrophotometry		Elemental analysis (copper content)
	405 nm	630 nm	
United States[a]	78.8–99.5	—	44.6–50.0
Elsewhere	39.2–53.5	26.5–34.2	22.8–35.9

[a] Data from ref. 7.

Since CCC was first marketed, little attention has been given to analytical methods to describe this preparation adequately. It is especially important now to be able to characterize CCC quantitatively because the current supply of this preparation in the United States is completely dependent upon foreign manufacturers using different technological protocols and raw material from various sources. It is hoped that our observations will stimulate methods development for this important biochemical entity.

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TECHNICAL COMMUNICATIONS

Determination of Reserpine in Tablets by Liquid Chromatography with Fluorescence Detection: Revised Procedure

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A procedure is presented for the determination of reserpine in tablets by liquid chromatography (LC) that is a slight modification of a method presented in a previous publication. The sample is extracted with methanol, and solutions are filtered through filter paper. For LC, a 7.5 cm column is used; the mobile phase is methanol containing a small volume of an aqueous solution of the sodium salt of 1-pentanesulfonic acid. Detection is by fluorescence with 280 nm excitation and 360 nm emission. Two commercial samples containing 0.1 and 0.25 mg reserpine were analyzed. For each sample, 2 determinations were made on a ground composite. Ten tablets were also analyzed individually. A linearity study was conducted, with solutions ranging in concentration from 80 to 120% of the amount present in the reference solution.

Reserpine, an alkaloid generally isolated from the root of *Rauwolfia serpentina*, is used as a mild hypotensive and sedative agent. Dosages in tablets currently sold are 0.1 and 0.25 mg. In a previous publication, a LC procedure was presented that employed a 30 cm normal phase column and detection by fluorescence (Cieri, U.R. (1985) *J. Assoc. Off. Anal. Chem.* **68**, 542–544). In the same article, several other methods for the analysis of this product were reviewed.

Over the past few years, we occasionally used the method. Several changes were introduced that permitted shorter analysis times and improved accuracy. A 7.5 cm column was preferred to the longer column; the mobile phase was modified by adding small volumes of an ion-pair solution to the methanol. Requirements were also specified for resolution, tailing factor, and reproducibility of replicate injections. Some changes were also made in the order and succession of injections of reference and of sample solutions. The revised procedure is described in this report.

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METHOD

Apparatus and Reagents

(a) *Reserpine.*—USP grade (United States Pharmacopeial Convention, Rockville, MD 20852), or equivalent.

(b) *Methanol.*—LC grade (Mallinckrodt, Inc., Science Products Div., St. Louis, MO 63134), or equivalent.

(c) *Filter paper.*—12.5 cm diameter, slow speed, No. 42 (Whatman LabSales, Hillsboro, OR 97123), or equivalent.

(d) *1-Pentanesulfonic acid, sodium salt.*—Aldrich Chemical Co., Inc., Milwaukee, WI 53201), or equivalent.

(e) *LC column.*—Novapak silica, 7.5 cm × 3.9 mm id (Millipore Corp., Bedford, MA 01730).

(f) *Injection valve.*—Equipped with 20 µL loop (Rheodyne, Inc., Cotati, CA 94931, or Alltech Associates, Deerfield, IL 60015).

(g) *Solvent delivery system.*—Model M-45 (Millipore Corp.), or equivalent.

(h) *Fluorescence LC detector.*—Model LS5 (Perkin-Elmer Corp., Instrument Div., Norwalk, CT 06859), or equivalent that can be set as follows: excitation 280 nm, emission 360 nm.

(i) *Recorder.*—Model R100 (Perkin-Elmer Corp.), or equivalent.

Solutions

(a) *Reserpine stock solution.*—Transfer 50.0 mg reserpine to 100 mL volumetric flask, add 1.0 mL chloroform, swirl to dissolve residue, dilute to volume with methanol, and mix.

(b) *Reserpine intermediate solution.*—Dilute 4.0 mL reserpine stock solution to 200.0 mL with methanol.

(c) *Reserpine reference solution, 0.0010 mg/mL.*—Dilute 10.0 mL reserpine intermediate solution to 100.0 mL with methanol.

(d) *System suitability solution.*—Transfer 0.20 mL reserpine stock solution to small beaker, and evaporate methanol to dryness. Add 1.0 mL chloroform to beaker, swirl to dissolve residue, and irradiate 10 min under longwave UV light. Evaporate chloroform to dryness, dissolve residue in warm methanol, dilute to 100 mL with methanol, and mix.

(e) *Salt solution.*—Dissolve 1 g 1-pentanesulfonic acid (sodium salt) in 50 mL water.

(f) *Mobile phase.*—Mix 4 mL salt solution (e) with 100 mL methanol. The volume of salt solution can be changed slightly, if necessary, to meet system suitability requirements.

(g) *Sample solutions.*—(*1*) *Ground composite assay.*—Weigh at least 20 tablets, calculate average tablet weight, and grind tablets to uniform powder. Transfer weighed amount of powder containing about 0.10 mg reserpine to 100 mL beaker, and add 50 mL methanol. Warm solution gently over hot plate or steam bath and stir with glass rod. Transfer solution to 100 mL volumetric flask, rinse beaker with small portions of methanol, and add rinse to flask. Cool solution to room temperature, dilute to volume with methanol, and mix. Filter solution through filter paper, and reject first 10 mL. (*2*) *Single tablet assay.*—Place tablet in 100 mL beaker, add 50 mL methanol, and warm solution gently over steam bath or hot plate. Carefully crush tablet with flat-bottom glass rod, and stir for a few minutes. Transfer to volumetric flask of appropriate size such that, when flask is filled to volume, resulting solution contains ca 0.0010 mg/mL. Continue as under (*1*), starting with words "Rinse beaker."

Procedure

System Suitability

Adjust flow so that reserpine elutes between 2 and 4 min. Inject system suitability solution. In addition to reserpine peak, chromatogram has 2 distinct peaks, both eluting earlier than reserpine peak. The resolution between reserpine and second degradation peak, as defined in United States Pharmacopeia XXII, p. 1565, was not less than 2.0.

Inject replicate aliquots of the reference solution. The tailing factor of the reserpine peak, as defined in USP XXII, p. 1567, was not greater than 2.0. The relative standard deviation of the responses in 4 consecutive chromatograms was not greater than 2.5%.

Sample Analysis

Inject the reference sample once, the sample solution twice, and then the reference sample again. If several samples are analyzed successively, continue in the same order, injecting the reference sample before and after each actual sample.

Average the responses of the reserpine peaks in the 2 sample chromatograms (R_u) and in the 2 reference chromatograms that precedes (R_s), and in the reference chromatogram that follows (R_s). Determine amount of reserpine in the sample solution by the following formula:

$$\text{Reserpine (mg)} = R_u \times 0.0010 \times \frac{V}{R_s}$$

where V is the volume of the sample solution in mL. For multisample analysis, as in the case of content uniformity, R_s could also be calculated as the average response of a larger number of reference chromatograms and applied to the determination of several samples, provided that the relative standard deviation for all the responses averaged does not exceed 2.5%.

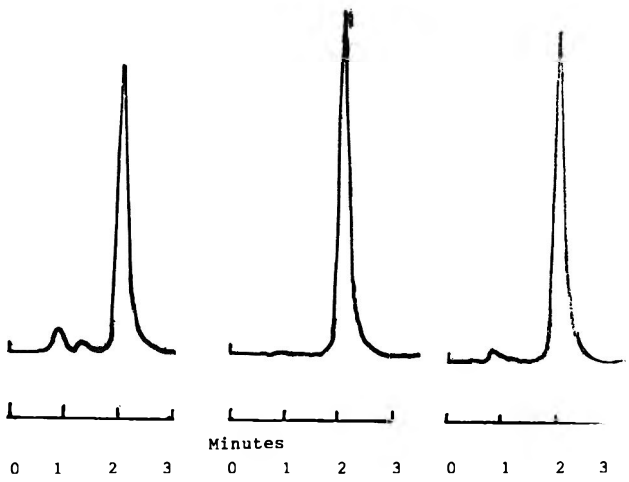

Figure 1. LC chromatograms: From left to right, system suitability, reference, and sample solutions.

Linearity Study

Transfer 8.0, 9.0, 10.0, 11.0, and 12.0 mL reserpine intermediate solution (**b**) to 5 separate 100 mL volumetric flasks, dilute to volume with methanol, and mix. Inject these 5 solutions separately as well as the reference solution, following order indicated under sample analysis. Calculate the amount of reserpine in each solution according to the following formula:

$$\text{Reserpine (mg)} = R_u \times \frac{0.1}{R_s}$$

Results and Discussion

The use of a shorter column (vs a longer column) permitted less consumption of mobile phase and a shorter time of analysis. Reserpine eluted after 2–4 min but was still adequately separated from its 2 main degradation products (Figure 1). The resolution between reserpine and the degradation peak that preceded it was not less than 2.0. The tailing factor of the reserpine peak was not greater than 2.0. The relative standard deviation, calculated based on the response of 4 consecutive injections of the reference solution, was not greater than 2.5%.

Table 1. Results of linearity study

Reserpine present, mg/100 mL	Reserpine found, mg	Recoveries, %
0.080	0.0780	97.5
0.090	0.0909	101
0.10	0.0995	99.5
0.11	0.112	102
0.12	0.120	100
Av. recovery		100
RSD, %		1.7

Table 2. Analysis of commercial tablets containing 0.1 and 0.25 mg/100 mL reserpine by proposed LC method

	Reserpine content on label, mg per tablet			
	0.1		0.25	
Commercial tablet	Reserpine found, mg	% of Labeled content	Reserpine found, mg	% of Labeled content
Ground composite assay				
1	0.0949	94.9	0.247	98.8
2	0.0945	94.5	0.249	99.6
Av.	0.0947	94.7	0.248	99.2
Single tablet assay				
1	0.0935	93.5	0.242	96.8
2	0.0922	92.2	0.248	99.2
3	0.0903	90.3	0.243	97.2
4	0.103	103	0.247	98.8
5	0.0948	94.8	0.248	99.2
6	0.0953	95.3	0.240	96.0
7	0.0937	93.7	0.245	98.0
8	0.0915	91.5	0.249	99.6
9	0.0908	90.8	0.256	102.4
10	0.102	102	0.254	101.6
Av.		94.7		98.8
RSD, %		4.7		2.0

It was observed, however, that sometimes (though not always) the responses changed slowly with time. As a result, the relative standard deviation became greater than 2.5% when the responses of a larger number of chromatograms were considered, especially when considerable time had elapsed. To eliminate, or at least to reduce, the error deriving from the variation in responses with time, the 'bracket system of injection' was followed; i.e., the reference was injected before and after each sample. This system was particularly indicated for multisample analysis, as in the case of content uniformity.

The reference solution contained 0.10 mg reserpine/100 mL; the concentration of drug in the sample solutions were expected to approximate that concentration. A linearity study was conducted with solutions whose concentrations ranged from 0.08 to 0.12 mg/100 mL solvent. Results are reported in Table 1. The method is probably linear even outside these ranges. However, if the concentration of reserpine in the sample solution is found to differ from that in the reference by more than 20%, it is advisable to prepare a new sample solution, so that the concentration of the sample will be closer to that of the reference.

Two commercial samples were analyzed for assay repeatability (Table 2, ground composite results) and content uniformity (Table 2, single tablet results). Results reported in Table 2 indicate a high degree of assay repeatability and uniformity of reserpine content among the 10 tablets tested.

TECHNICAL COMMUNICATIONS

Simple and Rapid Determination of Phytase Activity

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A simple and rapid method is described for determining the enzymatic activity of microbial phytase. The method is based on the determination of inorganic orthophosphate released on hydrolysis of sodium phytate at pH 5.5.

Phytase has been used successfully as a feed additive for poultry and pigs (monogastric animals) to improve phosphorus digestibility. Phytase is added to animal feed to decrease phosphorus excretion via manure. Different phytases can be found in nature (e.g., in seeds), and many microorganisms produce phytase (e.g., molds of the *Aspergillus* type). All these phytases can liberate orthophosphate from phytic acid, but the rate strongly depends on the conditions. A microbial phytase (product name, Natuphos) produced and marketed by Gist-brocades and BASF can release phosphorus from phytic acid under specified conditions. Natuphos shows optimal activity under the conditions present in the digestive tract of chickens and pigs. For application of phytase in the feed industry, a reliable and user-friendly method of determining phytase activity is needed.

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Published assays for phytase activity include determination of the rate of hydrolysis by using *myo*-inositol hexaphosphate (phytate) and *para*-nitrophenyl phosphate as substrate. Para-nitrophenyl phosphate as substrate has been described, but substrate selectivity studies revealed phytate to be the preferred substrate (1, 2).

Published colorimetric assays for the determination of inorganic orthophosphate are mostly based on the reduction of a phosphomolybdate complex by iron(II) sulfate or ascorbic acid to yield "molybdenum blues." Interference by phytate with the molybdenum blue methods (3) makes this determination less suitable.

With the application of phytase in mind, phytate was chosen as substrate and the official method of the European Community for phosphorus in feed (4) was chosen as the method for determining the released inorganic orthophosphate. No interference by phytate occurs with this method, which uses molybdovanadate as coloring reagent.

The purpose of the present study was to develop a simple, rapid analytical procedure that would provide reproducible phytase activities with good precision. On the basis of our experience with enzyme assays performed by different laboratories, we chose a relative method. In this way, minor differences from various sources can be avoided by relating the activities to a standard product with a known phytase activity. This activity is established by means of a calibration using an absolute method. For the calibration, a sufficient number of mutually independent analytical results are produced by (at present) 4 different laboratories. Following the procedure applied at Gistbrocades, the activity of the standard phytase is determined against phosphate on 3 different days with 2 substrate weighings per day, 2 standard weighings per substrate, and 2 determinations per weighing. Results are tested statistically and processed into an average activity. Time intervals for recalibration are laid down depending on the stability and/or the rate of use of the product.

METHOD

Apparatus and Reagents

(**a**) *Waterbath.*—Grant W28 (Grant Instruments, Cambridge, Ltd., Barrington, UK), thermostatically controlled to 37.0 ± 0.1°C by circulating water.

(**b**) *Spectrophotometer.*—Pye Unicam PU 8600 (Pye Unicam Ltd., Cambridge, UK), equipped with a 10.00 mm, continuous-flow cuvette with debubbler system (Cat. No. 179.010, Hellma GmbH & Co., Müllheim, Baden, Germany).

(**c**) *Centrifuge.*—Labofuge (Heraeus Christ, GmbH, Osterode, Germany), provided with rotor 03350 with inserts for 11 centrifuge tubes of 15 mL each and used at a relative centrifugal force of 3000 × *g*.

(**d**) *Water.*—Distilled water, or equivalent.

(**e**) *Buffer solution.*—Dissolve 1.76 g acetic acid (100%), 30.02 g $Na_2C_2H_3O_2 \cdot 3H_2O$, and 0.147 g $CaCl_2 \cdot 2H_2O$ (all analytical reagent grade; Merck, Darmstadt, Germany) in 900 mL water; adjust to pH 5.5 with acetic acid (100%), and dilute to 1 L with water.

(**f**) *Substrate solution.*—Dissolve 8.40 g sodium phytate ($C_6H_6Na_{12}O_{24}P_6 \cdot 10H_2O$) from rice (Cat. No. P-3168, Sigma Chemical Co., St. Louis, MO) in 900 mL buffer solution, adjust the pH to 5.5 with acetic acid (4 mol/L), and dilute to 1 L with water. Prepare this solution fresh daily.

(**g**) *Nitric acid solution.*—While stirring, slowly add 70 mL nitric acid (65%, analytical reagent grade, Merck) to 130 mL water.

(**h**) *Ammonium heptamolybdate stock solution.*—Dissolve 100 g ammonium heptamolybdate ($H_{24}Mo_7N_6O_{24} \cdot 4H_2O$) (analytical reagent grade, Merck) in 900 mL water, add 10 mL ammonia (25%), and dilute to 1 L with water. This solution may be kept at room temperature shielded from light for 1 month.

(**i**) *Ammonium vanadate stock solution.*—Dissolve 2.35 g ammonium vanadate (NH_4VO_3) (analytical reagent grade, Merck) in 400 mL water at 60°C. While stirring, slowly add 20 mL nitric acid solution, cool to room temperature, and dilute to 1 L with water. This solution may be kept at room temperature shielded from light for 1 month.

(**j**) *Color–stop mix.*—Mix 250 mL ammonium heptamolybdate stock solution and 250 mL ammonium vanadate stock solution. While stirring, slowly add 165 mL nitric acid (65%), (analytical reagent grade, Merck), cool to room temperature, and dilute to 1 L with water. Prepare this solution fresh daily.

(**k**) *Phytase standard.*—A commercial lot of phytase (Gistbrocades b.v., Delft) was selected as standard.

Preparation of Standard Solutions

Dilute weighed amounts of phytase standard in duplicate with buffer solution to prepare 200 FTU/mL stock solutions (stock solution A and B; FTU is defined in *Assay* section). Prepare working standards of, respectively, 0.02, 0.06, and 0.1 FTU/2 mL phytase standard in buffer solution by serial dilution of stock solution A and 0.04 and 0.08 FTU/2 mL phytase standard in buffer solution by serial dilution of stock solution B. Prepare the final dilutions in duplicate (standard and blank). Prepare standard stock solutions and dilute working standard solutions fresh daily. Analyze working standards as described below.

Preparation of Sample

Dilute weighed sample in duplicate (sample and blank) with buffer solution to a phytase activity within 0.02–0.08 FTU/2 mL solution to be analyzed.

Assay

(**a**) *Sample and standard solutions.*—Place the tubes to be incubated in the waterbath. Equilibrate each tube for 5 min. Add 4.00 mL substrate solution at 37.0 ± 0.1°C and mix. At time = 65 min, terminate the incubation by adding 4.00 mL color–stop mix and mix.

(**b**) *Blank.*—Place the tubes containing the blanks in the waterbath. Equilibrate each tube for 5 min. Add 4.00 mL color–stop mix, mix, and add 4.00 mL substrate solution to all blank tubes and mix.

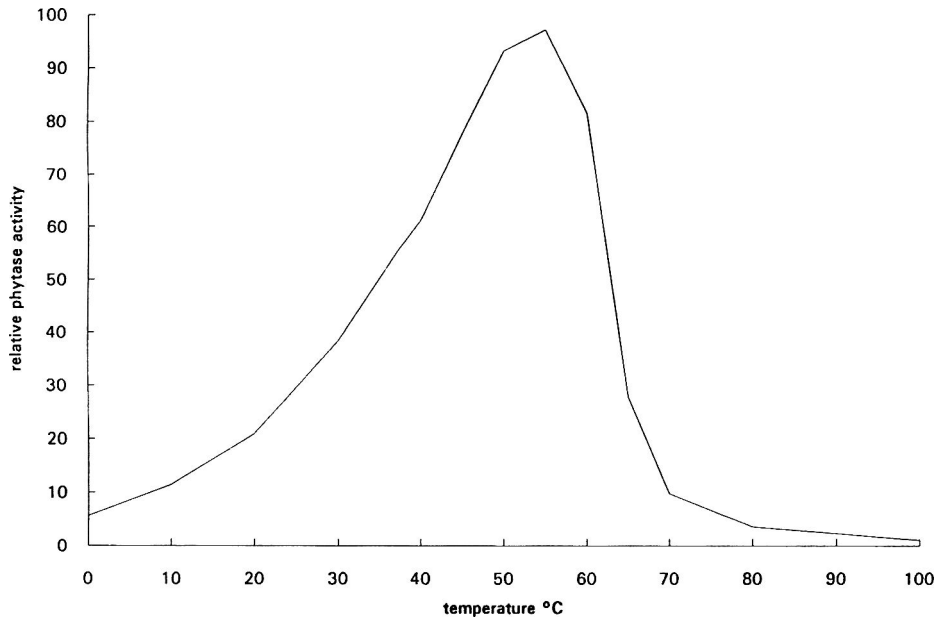

Figure 1. Temperature profile of Natuphos.

Centrifuge all tubes for 5 min. Measure the absorbance at 415 nm with the spectrophotometer after zeroing the instrument with water. Calculate the corrected absorbance difference by subtracting absorbance blank from that of the corresponding sample standard solution. On linear graph paper, plot the absorbance difference of the standard solutions against the corresponding exactly calculated activity (FTU/2 mL to be analyzed). Draw the best fitting curve through the origin. Determine the enzyme concentration by reading the corrected absorbance difference for the sample from the line produced. Enzyme activity is expressed in activity units (FTU); 1 FTU is the amount of enzyme that liberates 1 μmol inorganic orthophosphate/min under test conditions (pH 5.5; temperature 37°C; and substrate concentration, sodium phytate [$C_6H_6Na_{12}O_{24}P_6 \cdot 10H_2O$] at 0.0051 mol/L).

Results and Discussion

This paper describes the use of phytate as substrate for *Aspergillus niger* phytase and the use of molybdovanadate reagent to terminate the enzyme reaction and to measure the released free phosphate. Figure 1 is a plot of relative microbial

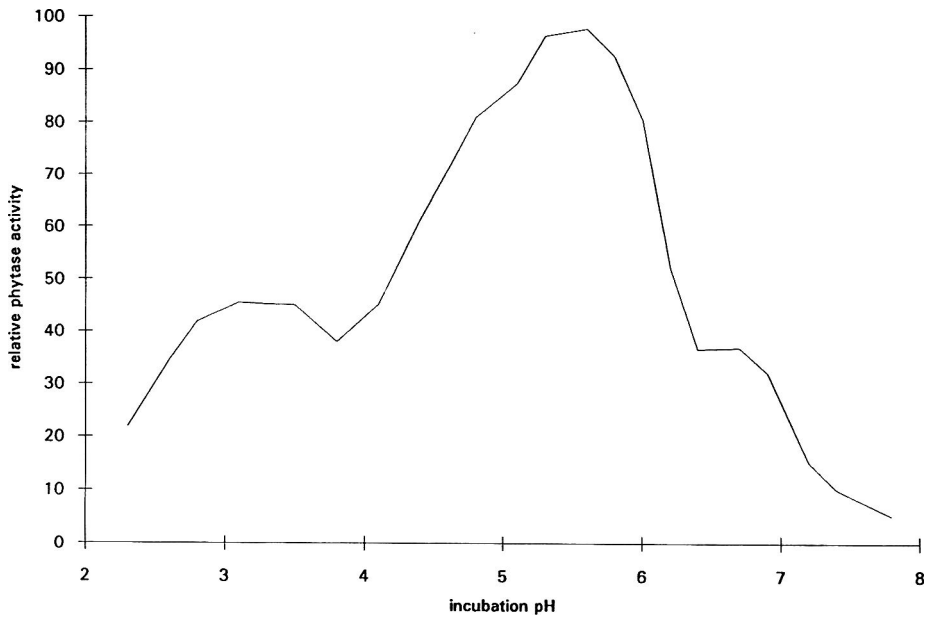

Figure 2. pH profile of Natuphos.

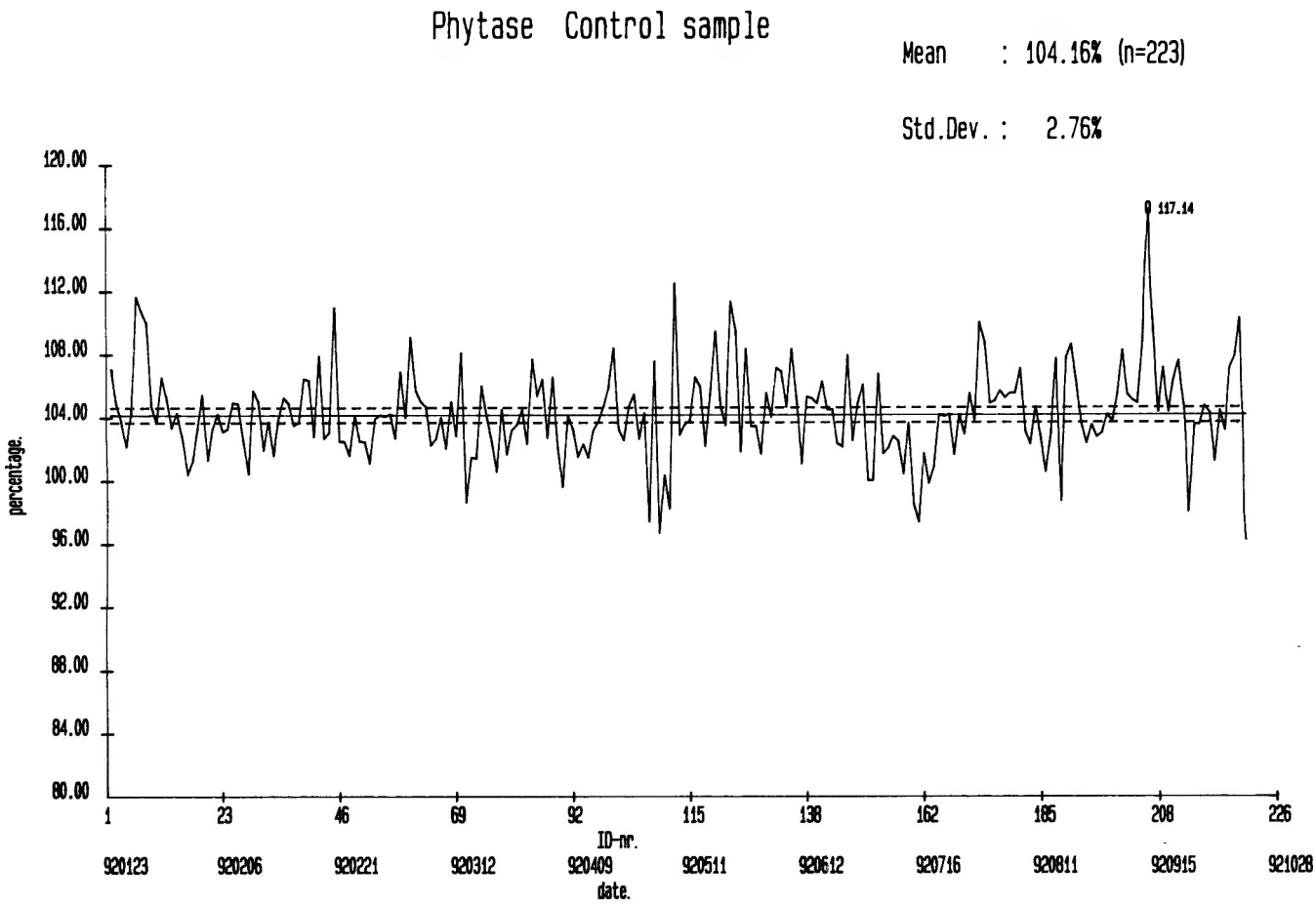

Figure 3. Phytase control sample.

activity versus incubation temperature. We chose an incubation temperature of 37°C, a compromise between the temperature conditions in the digestive tract and the temperatures available in analytical equipment for automated analysis. The incubation

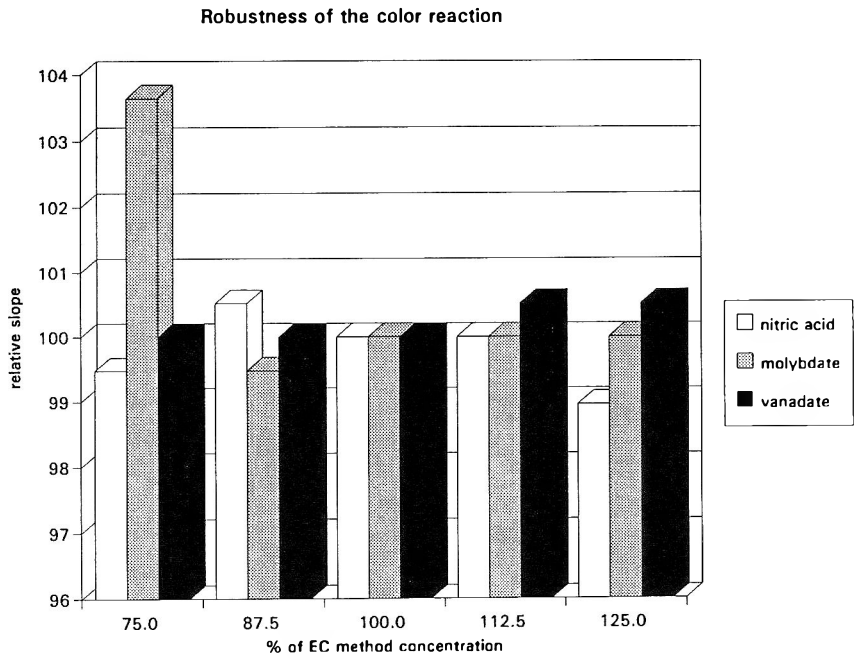

Figure 4. Robustness of color reaction.

Table 1. Determination of slope for phytase standard S54 (47000 FTU/g)[a]

Date	Slope, absorption per FTU per tube	
	Begin series	End series
Sept. 2, 1992	10.74	10.76
	10.84	11.02
Sept. 3, 1992	11.14	11.20
	11.47	11.49
Sept. 10, 1992	11.44	11.17
	11.34	11.04
Sept. 14, 1992	10.97	11.14
	11.40	11.34
Sept. 15, 1992	10.97	10.92
	11.23	10.92
Sept. 15, 1992	10.97	10.92
	11.23	10.92
Sept. 17, 1992	11.62	11.44
	11.03	10.86
Sept. 23, 1992	11.43	10.99
	10.99	10.69
Oct. 7, 1992	10.54	11.18
	10.76	11.07
Oct. 9, 1992	10.89	10.89
	10.67	10.79

[a] Average slope, 11.06; standard deviation, 0.26; relative standard deviation, 2.36%.

pH is an important factor in the production of free phosphate by phytase. Microbial phytase releases phosphate from phytic acid in a broad range of pH values. The optimum pH for microbial phytase is 5.5 (Figure 2). This optimum pH was chosen for the assay, although the enzyme reaction in the animal (digestive tract) takes place under several pH conditions. For almost a year, the described relative method for phytase activity has been executed at Gist-brocades and proved to be precise, reproducible, robust, and reliable (Figure 3). In the presence of substrate and free phosphate, molybdovanadate proved to be a robust reagent for variations (75 to 125%) in the concentration of all constituents (Figure 4). After the molybdovanadate reagent is added, a stable (for at least 1 h) yellow color is formed after 3 min.

The rate of formation of inorganic phosphate released from the substrate is linear for enzyme concentrations up to 0.10 FTU/2 mL (absolute absorbance should not exceed 1.0). With the phytase standard, the relative standard deviation for the slope was <2.5% ($n = 40$; Table 1).

In each series, a control sample with known phytase activity was determined. To establish the phytase activity, the same calibration procedure used for the phytase standard was applied. The overall relative standard deviation for this control was <3% ($n > 100$).

The precision in enzyme preparations (phytase activity >1000 FTU/g, e.g., Natuphos) was approximately 2.5%. For feed samples (phytase activity <1 FTU/g), because of inhomogeneity, a value of 5 to 10% was more realistic. Although the accuracy was close to 100% for all matrixes examined, the presence of enzyme activators or inhibitors in unknown sample matrixes can affect the slope of the curve for the proposed assay. In such circumstances, matrix matching or the use of standard addition is essential. The limit of detection was 0.004 FTU in the measured solution ($3 \times \sigma_{blank}$, $n = 10$).

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TECHNICAL COMMUNICATIONS

Analysis of Streptomycin and Dihydrostreptomycin in Milk by Liquid Chromatography

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A method developed for the determination of the aminoglycoside antibiotics streptomycin and dihydrostreptomycin in tissues was applied to the analysis of fluid milk. Samples are extracted with 3.6% perchloric acid, and then injected onto a trace enrichment column, from which they are eluted onto a reversed-phase analytical column. The analytes are detected by fluorescence following post-column derivatization with 1,2-naphthoquinone-4-sulfonic acid. Recovery of analytes was in the range of 50–65% for skim or partially defatted fluid milk, while recoveries for homogenized whole milk were lower. Limits of quantitation were 10 ppb for streptomycin and 20 ppb for dihydrostreptomycin.

In the United States, 6 injectable products containing dihydrostreptomycin (DHS) in combination with penicillin G for the treatment of mastitis and other diseases in dairy cattle have been listed in the Food Animal Residue Avoidance Data Bank Trade Name File (1). A recently published compendium also lists 6 such formulations approved for similar use in Canada (2). In addition, one formulation containing only DHS has been approved for treatment of dairy cattle in Canada, where a 96 h discard period for milk is required for approved formulations containing DHS. A similar discard period is mandated in the United States for milk from cows that have been treated with DHS. Approved formulations containing streptomycin (STR) are intended for use as additives to feed or water and are not intended for use in lactating dairy cattle (1, 2).

No sensitive chromatographic methods for STR and DHS in milk have been reported to date. We have, therefore, investigated the application of a method recently developed in our laboratory for the analysis of STR and DHS in animal tissues to the analysis of these residues in milk (3). The method is based, in part, on the use of ion-pairing liquid chromatography (LC) and post-column derivatization with β-naphthoquinone-4-sulfonate (NQS), as originally reported for the determination of STR in serum samples by Kubo et. al. (4).

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Presented in the Symposium *Milk: Antibiotics and Other Contaminants*, 106th Annual AOAC International Meeting and Exposition, Cincinnati, OH, August 31–September 2, 1992.

METHOD

Reagents

(a) *Acetonitrile.*—LC grade (absorbance <0.02 at 210 nm).

(b) *Water.*—Obtained from a Barnstead RO/Nanopure ultrafiltration unit.

(c) *Streptomycin sulfate (STR) and dihydrostreptomycin sulfate (DHS) standards.*—Sigma Chemical Co., St. Louis, MO.

(d) *1-Hexane sulfonate, sodium salt.*—Supelco Canada Ltd., Oakville. ON, Canada.

(e) *1,2-Naphthoquinone-4-sulfonic acid, potassium salt (NQS).*—Aldrich Chemical Co., St. Louis, MO.

(f) *Perchloric acid.*—69–72% reagent grade.

(g) *Perchloric acid, 3.6%.*—Add 50 mL perchloric acid to 500 mL water; mix and dilute to 1 L.

(h) *Ion-pair concentrate for sample preparation.*—0.2 M 1-hexanesulfonic acid. Dissolve 3.76 g 1-hexanesulfonic acid in water and dilute to volume in 100 mL volumetric flask.

(i) *Mobile phase.*—Water–acetonitrile mixture (83 + 17) containing 10 mM 1-hexanesulfonic acid and 0.4 mM NQS at pH 3.3. Dissolve 1.88 g 1-hexanesulfonic acid and 0.11 g NQS in 830 mL water, add 170 mL acetonitrile, and adjust to pH 3.3 with glacial acetic acid. Filter through a 0.45 μm membrane unit. Prepare fresh daily and store in an amber flask during use.

(j) *LC loading solution.*—10 mM 1-hexanesulfonic acid at pH 3.3. Dissolve 1.88 g 1-hexanesulfonic acid in 1 L of water, adjust to pH 3.3 with glacial acetic acid, and filter through 0.45 μm membrane unit prior to use.

(k) *Post-column reagent for LC analysis.*—0.5 M sodium hydroxide. Dissolve 20 g sodium hydroxide in water and dilute to 1 L.

Apparatus

(a) *LC equipment.*—Mobile phase and loading solution pump, Waters Model 510, with automated switching valve for column selection (Waters Chromatography Division, Mississauga, ON, L4V 1M5, Canada); Rheodyne 7125 sample injection valve with 2 mL loop (Supelco Canada Ltd.); sample enrichment column, 4 cm × 4.6 mm id, packed by the viscosity method with Inertsil 5 μm C-8 packing (Lab Link Inc., 2211 Jonquil, Rockford, IL); analytical column, 25 cm × 4.6 mm id packed with 5 μm LC-8-DB (Supelco Canada Ltd.); PCX-3000 post-column reaction module with 2 mL reaction coil

(Pickering Laboratories, 1951 Colony St, Mountain View, CA); Spectroflow 980 fluorescence detector (Kratos Analytical Instruments [now Applied Biosystems Inc.], Foster City, CA); Kipp & Zonen Model BD 41 strip chart recorder (Mandel Scientific Co., Ltd., Guelph, ON, Canada).

(b) *Centrifuge.*—IEC Centra-8 general purpose centrifuge (Fisher Scientific Ltd., Nepean, ON, Canada).

Determination

(a) *Preparation of standards.*—(*1*) Stock standard (500 µg/mL STR, 1000 µg/mL DHS): dissolve 65 mg STR and 125 mg DHS in water and dilute to 100 mL. (*2*) Working standard (5 µg/mL STR, 10 µg/mL DHS): dilute 1 mL of stock standard to 100 mL with water. (*3*) LC standards (10/20, 20/40, 50/100 ppb STR/DHS): dilute 20, 40, and 100 µL of working standard to 10 mL with LC loading solution.

(b) *Sample extraction.*—Weigh 10 g of milk into a polypropylene centrifuge tube and add 3 mL of 3.6% perchloric acid. (Milk samples used for recovery studies were spiked with STR and DHS prior to the addition of the perchloric acid.) Shake this mixture for 30 s on a horizontal shaker and then centrifuge at 2000 × *g* for 5 min. Decant the supernatant into a 10 mL volumetric flask, add 70 µL 5N sodium hydroxide and 500 µL ion-pair concentrate, and dilute to volume with water.

(c) *Chromatographic determination.*—Chromatography is performed using the chromatographic system with column-switching valving as previously described (3) with the following conditions: mobile phase flow rate, 1.5 mL/min; loading solution flow rate, 1.0 mL/min; injection volume, 2 mL; post-column reagent flow rate, 0.5 mL/min; post-column reaction temperature, 50°C; fluorescence detection parameters, 365 nm excitation, 418 nm emission, range 0.05.

Table 1. Recoveries of STR and DHS from fluid milk[a]

Sample	STR rec., %	CV, %	DHS rec., %	CV, %
Skim milk	56.2	9.3	50.2	9.0
2% Milk	65.0	7.6	59.1	5.6
Homogenized milk	32.6	6.8	34.8	8.1

[a] Average of 6 samples fortified with 20/40 and 100/200 ppb of STR/DHS.

Draw approximately 2.5 mL of extracted sample into a 3 mL syringe and place a 25 mm, 0.45 µm filter on the syringe tip. Attach the syringe/filter assembly to the injector, and load the sample into the 2 mL injection loop. With the column selection valve in position 1, switch the injector valve to inject to allow the sample to be loaded onto the enrichment column. Flush this column with loading solution for 5 min to elute co-extracted materials to waste, while retaining the analytes on the column. Switch the column selection valve to position 2 to allow the loading solution to be pumped to waste and to elute the analytes with the mobile phase from the enrichment column to the analytical column. After 5 min of rinsing, return the column selection valve to position 1 to prepare the enrichment column for another injection, while the mobile phase continues to pass through the analytical column. Automated switching of the column selection valve is controlled by a timed program sequence on the fluorescence detector.

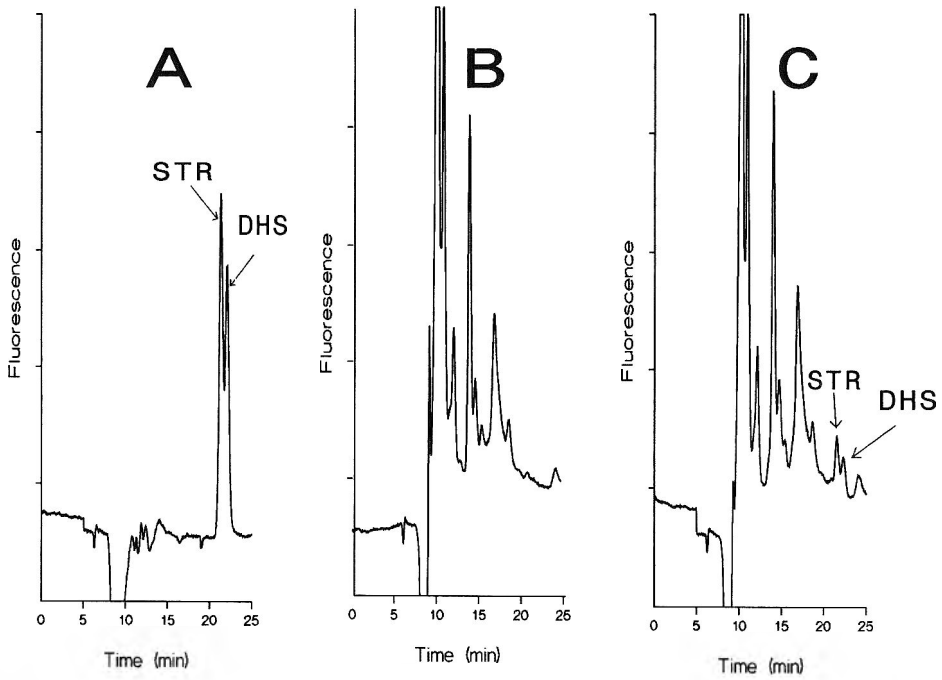

Figure 1. Typical chromatograms for the analysis of streptomycin (STR) and dihydrostreptomycin (DHS): A, mixed standard (100 ppb STR, 200 ppb DHS); B, blank 2% fat milk; C, spiked 2% fat milk (10 ppb STR, 20 ppb DHS). Time units on horizontal axes refer to retention times using a mobile phase with an 83:17 aqueous/organic ratio.

Results and Discussion

This method was originally developed for use with tissue and has been modified for use in milk, mainly by the elimination of a cleanup step, which used ion-exchange chromatography, to remove a matrix interference in tissue samples (3). This interference was not encountered in fluid milk products. Otherwise, the perchloric acid extraction and chromatographic analysis paralleled those previously developed for tissue samples.

Recoveries of STR/DHS spiked in milk samples at levels of 20/40 and 100/200 ppb are listed in Table 1. Homogenized milk yielded significantly lower recoveries than the reduced fat milk samples that were also tested. It was observed that the volume of the perchloric acid extract obtained was reduced as the fat content of the milk sample increased.

The chromatograms shown in Figure 1 for milk containing 2% fat are typical of those obtained for all types of milk analyzed. Because it was considered unlikely that both STR and DHS would be encountered in the same sample, a mobile phase composition was chosen that, while permitting the identification of each drug, minimized the analytical time required for samples containing only one of these antibiotics. The chromatogram of a standard solution containing 100 ppb STR and 200 ppb DHS shows that baseline resolution has not been achieved under the chromatographic conditions used. Should a sample be found to contain both drugs, the mobile phase may be adjusted to afford full resolution for improved quantitation. Using our column, such improved resolution could be obtained by increasing the aqueous/organic ratio of the mobile phase from 83:17 to 85:15, but this change also resulted in retention times of approximately 40 min for the analytes.

It is essential that a proper post-column reaction system be assembled to produce a reasonable baseline. The reaction pump must be equipped with a pulse dampener, and the reaction coil must be maintained at a steady temperature free from fluctuation. Otherwise, an unstable baseline that impedes the analysis results.

Attempts to apply the method developed for fluid milk to processed milk products, such as yogurt and cottage cheese, proved unsuccessful. The chromatograms of these products contained interfering matrix components that co-eluted with STR and DHS. Further sample cleanup, using solvent extraction or the ion-exchange technique used for tissue samples (3) may be required to apply this approach to the analysis of other milk products.

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TECHNICAL COMMUNICATIONS

Voltametric Behavior of Zopiclone: Polarographic Determination in Tablets

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Electrochemical reduction of zopiclone at different pHs and concentrations was studied by polarography and cyclic voltametry. Both techniques revealed a reduction process with weak adsorption of both zopiclone and its reduction derivative. Zopiclone exhibited 2 differential pulse polarographic peaks at the dropping mercury electrode. The first peak was used to develop a differential pulse polarographic analytical procedure for determining the drug in pharmaceutical dosage forms. Reproducibility and recovery coefficients of variation were 1.6 and 2.2%, respectively. Analysis of commercial zopiclone tablets showed uniformity in zopiclone content. The method is simple and rapid because separation of excipients is unnecessary.

Zopiclone {6-(5-chloro-2-pyridyl)-7-[(4-methyl-1-piperazinyl)carbonyloxy]-6,7-dihydro[5*H*]pyrrolo[3,4]pyrazin-5-one} (Figure 1) is a new nonbenzodiazepine anxiolytic drug for patients suffering from insomnia. It has hypnotic properties (1), rapid onset of action, and few associated side effects (2, 3). The optimum effective daily oral dose of zopiclone is 7.5 mg (4, 5), and pharmacokinetic studies show that levels in plasma are usually 20–80 ng/mL.

Zopiclone undergoes hepatic metabolism that includes demethylation, oxidation, and decarboxylation. Its pharmacological properties were reported (8).

Methods to detect zopiclone all involve liquid chromatography (LC) with spectrofluorometric detection (9–12) and are applied to plasma samples. The detection limit is 2–5 ng/mL. No analytical assays for pharmaceutical forms of zopiclone have been reported to date.

Modern computer-based electrochemical instrumentation has increased the usefulness of electrochemical methods such as differential pulse polarography and square-wave voltametry. Several electrochemical determinations of drugs in pharmaceutical forms have shown important advantages of these methods (13, 14).

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The electrochemical behavior of zopiclone was investigated and a differential pulse polarographic procedure was developed for zopiclone in its pharmaceutical forms.

Experimental

Apparatus

(**a**) *Polarograph.*—Tacussel assembly operated in direct current (dc) and differential pulse polarographic (dpp) mode consisting of an EPL-3 recorder equipped with a TI-PULS module, similar to one previously described (15). Operating conditions: pulse amplitude, 60 mV; potential scan rate, 5 mV/s; drop time, 1 s; voltage range, –0.4 to –1.3 V; current range, 1.25–5.0 μA; temperature, 25°C.

(**b**) *Polarographic cell.*—Tacussel CPRA measuring cell with dropping mercury electrode, platinum wire counter electrode, and saturated calomel reference electrode.

(**c**) *Cyclic voltametry.*—Linear-sweep cyclic voltametry carried out in computer-based INELECSA assembly Model PDC-210, similar to that described in literature (16). The working electrode was a hanging mercury drop electrode, Metrohm Model EA-290.

Reagents

All chemicals were analytical grade. Double-distilled water was used.

(**a**) *Universal buffer.*—Dissolve 12.11 g of tris(hydroxymethyl)aminomethane, 13.61 g of KH_2PO_4, 6.18 g of boric acid, 21.01 g of citric acid, and 7.46 g of KCl in distilled water, and then dilute to 1 L (pH range, 2.0–12.0). Adjust with HCl to desired pH.

(**b**) *Zopiclone standard solutions.*—Accurately weigh 24.3 mg of zopiclone standard (99.8% purity; Rhodia Merieux Laboratories, Santiago, Chile); dissolve and dilute with universal buffer (pH 3.5) to 25 mL (concentration, 2.5mM).

(**c**) *Solutions for polarographic calibration curve.*—Dilute the standard solution accurately with universal buffer (pH 3.5) to obtain solutions ranging from 0.02 to 0.2mM.

(**d**) *Synthetic samples.*—Prepare excipient powders for recovery studies according to manufacturer's batch formulas for 5.0, 7.5, and 10.0 mg of zopiclone. The following excipients were used: methylcellulose, lactose, corn starch, Mg stearate, and $NaHPO_4$.

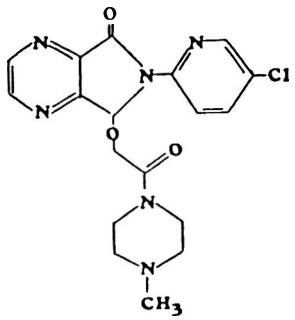

Figure 1. Molecular structure of zopiclone.

Sample Preparation

Dissolve a tablet or synthetic sample containing ca 5.0, 7.5, or 10.0 mg of zopiclone in 100 mL universal buffer adjusted to pH 3.5, sonicate for 5 min, and dilute to 200 mL in a volumetric flask with buffer.

Polarographic Determination

Transfer ca 25 mL of the sample solution to a dry polarographic cell, and then deaerate by bubbling nitrogen through the solution for 10 min. Scan sample solution by differential pulse polarography from –0.4 to –0.7 V. Calculate zopiclone concentration from a standard calibration curve measured under the same conditions.

Results and Discussion

Electrochemical reduction of zopiclone gave 2 differential pulse polarographic peaks at the dropping mercury electrode. In acidic media, the first peak (peak I) appeared between –400 and –600 mV, and the second peak (peak II) was between –950 and –1200 mV, depending on pH. Both peaks were resolved in differential pulse mode; however, when direct current polarography was used, only the first wave was resolved adequately (Figure 2). The polarographic response was strongly pH dependent. For both peaks, peak potentials shifted with increasing pH to more negative potentials and showed linear dependence (Figure 3).

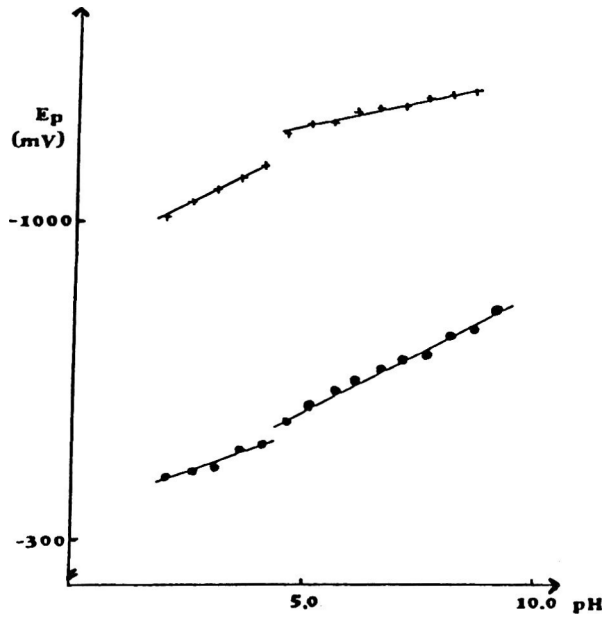

Figure 3. Linear relation between differential pulse polarographic potential peak and pH: ●, peak I; +, peak II.

The lines show a break at approximately pH 4, indicating a change in the reduction mechanism, probably resulting from a change in the protonation of the molecule. We have ascribed this change to an apparent polarographic dissociation resulting from the pyridine nitrogen in the molecule. Similar pK_a values for related compounds were reported in the literature (17).

Peak heights also were pH dependent (Figure 4). At pH values lower than 4, both peak heights were independent of pH; however, starting at pH 4, the height of peak II decreased with increasing pH, and the height of peak I decreased slightly and remained pH independent. The dramatic pH effect on the height of peak II implies dissociation in the electroactive group.

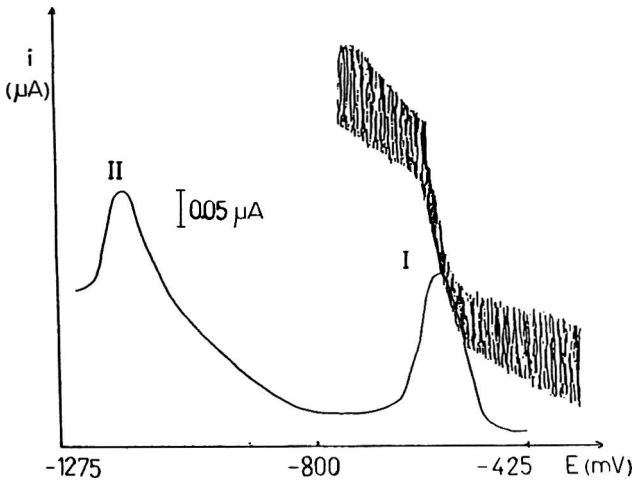

Figure 2. Differential pulse polarographic peaks and dc polarographic wave of 1×10^{-4}M zopiclone solution at pH 3.5.

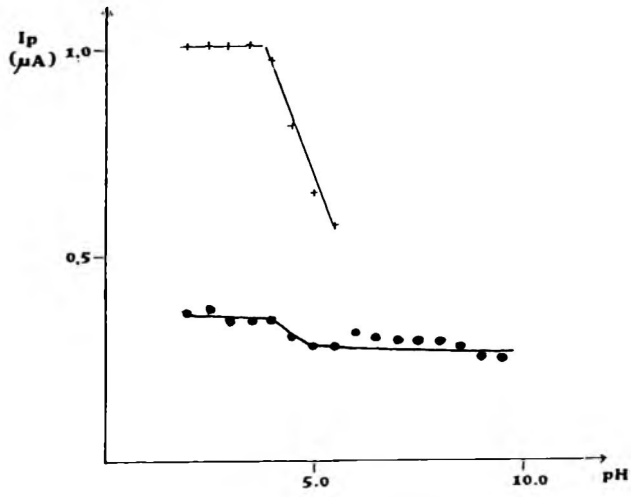

Figure 4. Peak current versus pH: ●, peak I; +, peak II.

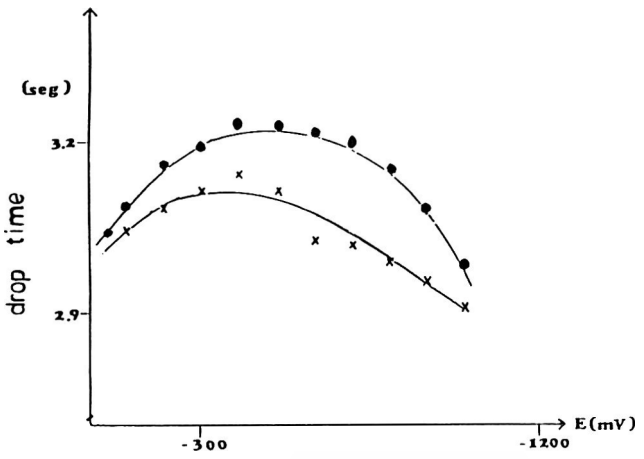

Figure 5. Electrocapillary curve: ●, buffer solution; x, 1 × 10^{-4}M zopiclone in buffer solution.

Because peak II overlapped with the supporting electrolyte discharge, peak I was studied more thoroughly. To understand the nature of the electrode process that controls the limiting current of the first peak i_1, we studied the limiting current behavior versus the height of the mercury column, temperature, and drop time. We obtained a linear relation between the limiting current and the square root of the height of mercury column (corrected for back pressure) and a temperature coefficient of 2.07%/°C. Both results support a diffusion-controlled process. However, we obtained a $(d \log i_1)/(d \log t)$ value of 0.11, which is different from the theoretical value of 0.19 for a pure diffusion-controlled process (18). The difference is probably due to an adsorption phenomenon. Electrocappillary curves (Figure 5) confirm this hypothesis by showing that zopiclone or its reduction product is adsorbed on the dropping mercury electrode.

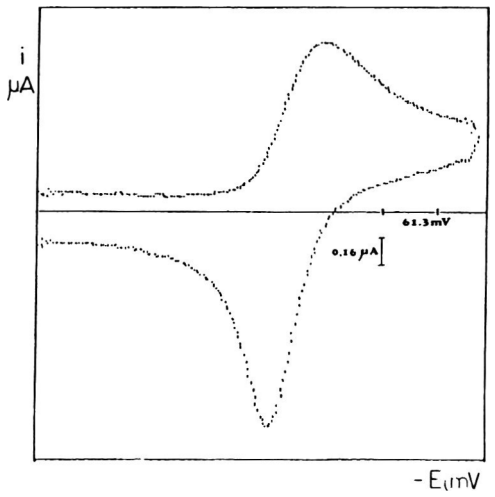

Figure 6. Cyclic voltamogram of 1 × 10^{-4}M of zopiclone in buffer (pH 3.5): sweep rate, 0.8 V/s; initial potential, –250 mV; cathodic peak, –573.5 mV; anodic peak, –513.8 mV.

Figure 6 shows the cyclic voltamogram corresponding to peak I of zopiclone. The shape of this voltamogram shows that the reduction process is reversible; the sharpness of the peaks indicates adsorption (19). Furthermore, the ΔE_p ($\Delta E_p = E_{pb} - E_{pf}$, where E_{pb} and E_{pf} are backward and forward peak potentials, respectively) value of 60 mV indicates that the electrode reaction involves the transfer of 1 electron. The plots of current function, $i_p/(v^{1/2}c)$ (where i_p = peak height, v = sweep rate, and c = concentration), versus the square root of the scan rate for both anodic and cathodic peaks (Figure 7) are conclusive evidence of weak adsorption.

The scan rate is probably the most important experimental parameter used to differentiate the effects from adsorbed reactant and from material arriving at the electrode by diffusion; adsorbed material constitutes a fixed amount of material (time independent), whereas the amount of diffusing material is time dependent. Therefore, varying the time window in the voltametric experiment permits us to clearly differentiate these effects. Figure 8 exhibits a sequence of cyclic voltamograms at different scan rates. When the scan rate was increased, the ad-

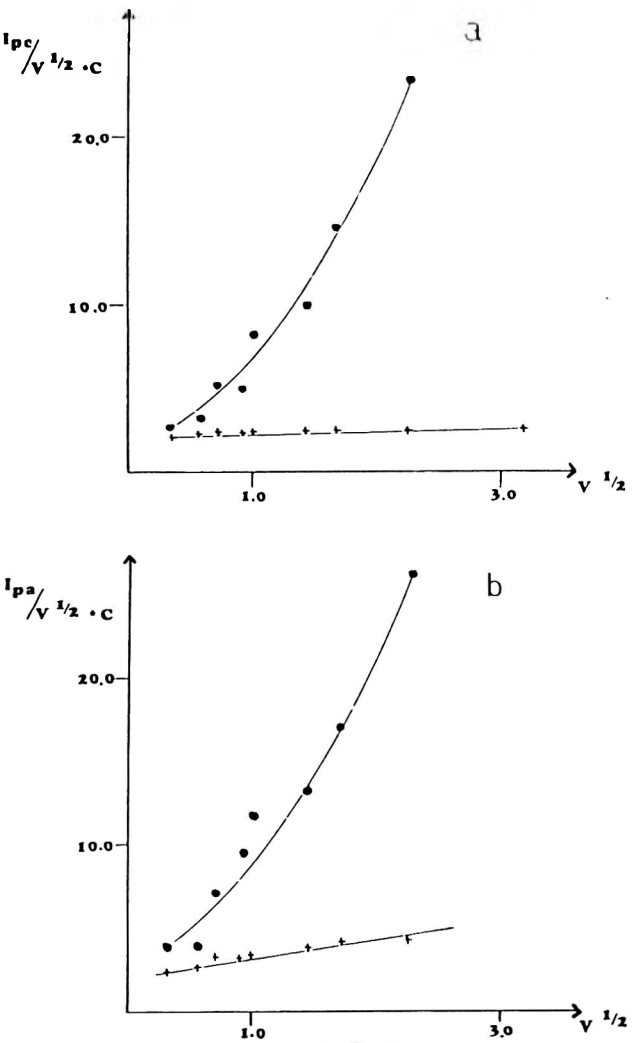

Figure 7. Current function $i_p/v^{1/2}c$ versus square root of sweep rate at 2 concentrations: (a) cathodic current; (b) anodic current: +, 1 × 10^{-3}M; ●, 1 × 10^{-4}M.

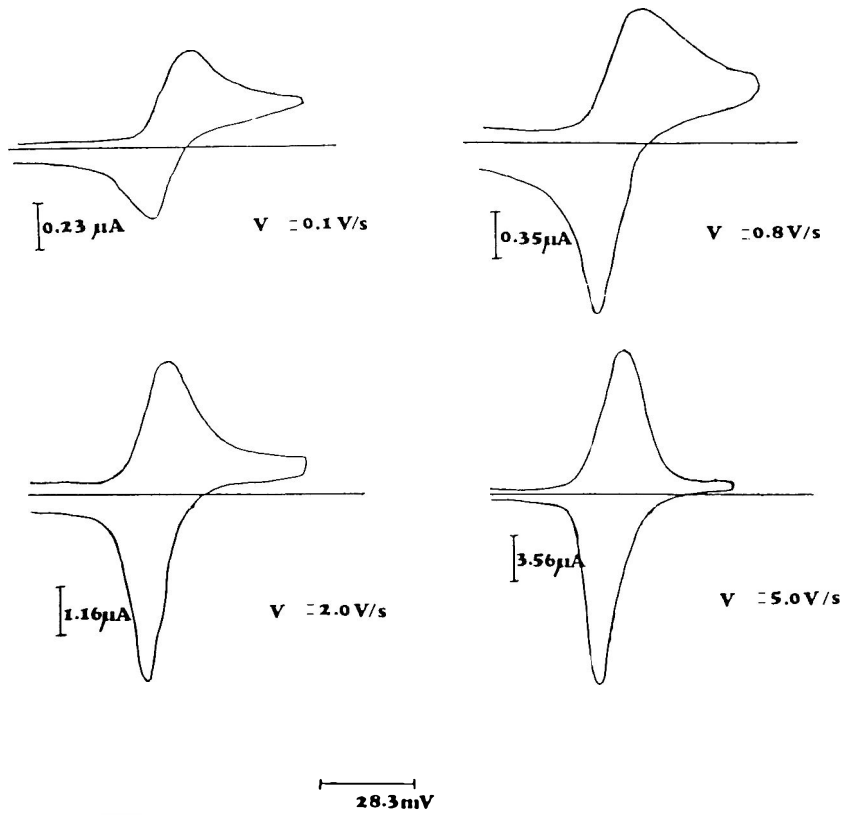

Figure 8. Evolution of the adsorptive character of the cyclic voltamograms at different sweep rates.

sorptive character (sharpness of peaks) of both peaks also increase.

In conclusion, the cyclic voltametric experiments indicate that both zopiclone and its reduction product are weakly adsorbed to the mercury electrode. Zopiclone reduction turns out to be a very good model when the electroactive species and its reduction product are both weakly adsorbed, according to the theory developed by Wopschall and Shain (19).

By considering the polarographic and cyclic voltametric experiments, we were able to propose a mechanism for the reduction of zopiclone. The pyrazine and pyridine rings are 2 potential reduction electroactive groups (Figure 1). Peak potential values for peak I (<1 V) were similar to values for other related pyrazine derivatives (16, 20, 21), and we ascribed this peak to the reduction of the pyrazine ring. Peak II, obtained at more cathodic potentials, probably results from the reduction of the pyridine ring that has been shown to occur in related pyridine-containing compounds (22). The process leading to peak I is 2-electron, 2-proton reduction of the pyrazine moiety to form the corresponding dihydropyrazine derivative (21). The fact that the cyclic voltametric behavior gives rise to a ΔE_p of 60 mV implies that the 2-electron reduction occurs via 2 monoelectronic steps with identical energy requirements, thus producing 2 overlapping monoelectronic peaks. This behavior was observed previously for pyrazine (20).

For quantitative purposes, we selected peak I by differential pulse polarographic mode at pH 3.5. Under these conditions we obtained a linear relation between peak current (i_p) and zopiclone concentrations less than 2×10^{-4}M. At higher concentrations, linearity was lost, and current values obtained were lower than those predicted by Ilkovic's equation (Figure 9). This behavior is due to adsorption during the electrode process. To quantify the drug, we used the calibration curve method

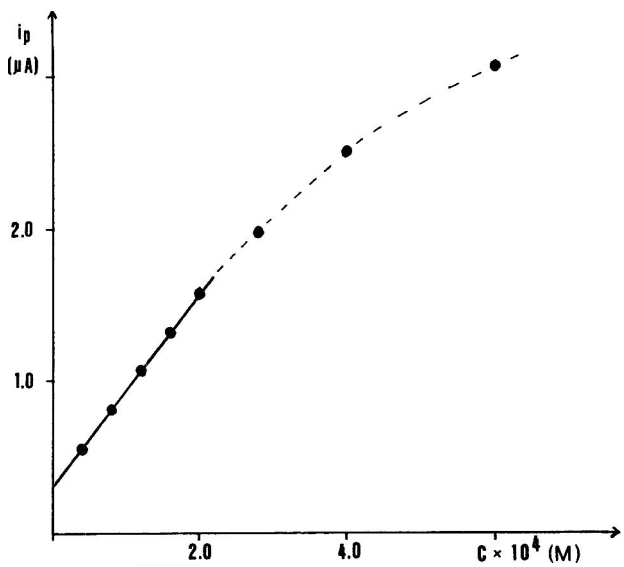

Figure 9. Relation between peak current (i_p) and zopiclone concentration.

Table 1. Recovery of zopiclone added at different levels[a]

Sample No.	Recovery, %		
	5.0 mg added	7.5 mg added	10.0 mg added
1	104.0	99.0	97.0
2	98.0	103.0	98.2
3	101.0	104.0	103.0
4	96.0	100.1	97.5
5	101.0	98.5	99.2
6	100.2	98.6	98.0
7	99.2	104.0	99.0
8	97.5	100.1	102.0
9	102.0	98.5	98.4
10	98.8	98.6	99.5
Mean	99.8	100.4	99.2
Standard deviation, %	2.35	2.32	1.92
Coefficient of variation, %	2.35	2.31	1.94

[a] Each synthetic mix was prepared containing 5.0, 7.5, and 10.0 mg of zopiclone pure drug plus excipients according to manufacturers' instructions.

Table 2. Individual tablet[a] assay for zopiclone (declared amount, 7.5 mg)

Sample No.	Zopiclone found, mg	Percentage of declared amount
1	7.6	102.0
2	7.2	96.7
3	7.5	100.9
4	7.5	100.9
5	7.9	104.8
6	7.5	100.8
7	7.6	102.0
8	7.5	100.8
9	7.6	102.0
10	7.5	100.0
Mean	7.5	
Standard deviation	0.17	
Coefficient of variation, %	2.27	

[a] Imovane; Rhodia Merieux, Santiago, Chile.

with concentrations between 0.1 and 0.01mM. The calibration curve equation was as follows:

$$i_p = (6.38\ \mu A/mM)C + 0.29\ \mu A$$

where i_p is the peak current (μA) and C is the zopiclone concentration (mM). The correlation coefficient was 0.998 for 10 points between 1×10^{-5} and 1×10^{-4}M. The reproducibility of the method was calculated from 10 independent runs, and a coefficient of variation of 1.6% was obtained. To check the accuracy and precision of the developed method, we carried out a recovery study. From the results (Table 1), we concluded that the method was sufficiently accurate and precise for analysis of pharmaceutical forms. Recoveries of zopiclone from a synthetic mix at different levels show the validity of the method. Table 2 shows the amounts of zopiclone found in commercial tablets declared to contain 7.5 mg of zopiclone. Sample preparation was easy, because the excipients did not interfere in the analysis. Consequently, neither separation nor extraction procedures were required. The proposed method is a very good alternative for routine determinations in quality control laboratories.

Acknowledgments

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AOAC SYMPOSIUM: AUTOMATED GOOD LABORATORY PRACTICE

Automated Analysis with Computerized Information Transfer in a University Hospital

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The organization of a central clinical chemistry laboratory of a university hospital, which in 1992 analyzed 880 000 results, is presented. Automation with midsize selective analyzers, which run in parallel and are able to do positive sample identification on the primary bar-coded tubes, in connection with a laboratory computer system and a central hospital computer allowed for reliable and timely obtainment of results. The output of the results is made on laser printers in the medical wards, thus alleviating the pneumatic sample transport systems. Assessment of the turnaround time show that it can be kept below one hour. Such a setup without sorting out emergency demands allows a simplified sample flow. Thus, there is no need for a laboratory to make "stat" requests.

The task of a clinical chemistry laboratory is to produce reliable results promptly for a clinician who seeks to test a hypothesis and to draw therapeutic consequences. The increasing financial pressure and the progressive shortening of hospitalization time are incentives for rendering the laboratory organization more efficient and cost effective.

As shown in Figure 1, the work load of the Central Clinical Chemistry Laboratory (LCC) of the University Hospital of Lausanne (1137 beds) has increased since 1984, while the number of technicians (31.8 positions) and of academic staff (n = 7) was kept constant. Automation had to be enhanced, and electronic data processing and a reorganization of the work flow had to be undertaken. We present the results, which led us to abolish that "stat" laboratory, and therefore, to streamline the processing, to use midsized selective analyzers in parallel with positive sample identification, and to alleviate the work load by printing the results in the wards after electronic data transfer.

Equipment and Method

The University Hospital uses a central computer system (Unisys/Bourroughs) with the Bourrhoughs Hospital Information System (BHIS) software for archiving patients' administrative data (including billing). The results are stored from the central laboratories. Since 1989, the Central Hematology Laboratory and the LCC have shared a laboratory computer system (Swisslab 2020, Frey, Berlin) that is linked to BHIS. The computer system is equipped with an HP A900 and an HP A600 CPU (16 MB RAM) and 2 mirror disks (443 MB each). The clinical chemistry laboratory to which we restrict the further presentation is equipped with 24 terminals, 16 printers, bar code readers, and thermoprinters for duplicating bar codes when needed. Several instruments are attached to the Swisslab system by bi-directional connections (for the main work load: 3 Hitachi Model 717s, with positive sample identification; 1 Hitachi, Model 704 for urine analysis). The LCC fulfills demands of the hospital wards and the dialysis, pediatric, and intensive care units 24 hours a day. The only exceptions are those infrequent analyses for which prior negotiation with a staff member is required if the result must be reported during the night or weekend.

Before reorganizing the laboratory, all the request forms were collected during one week; this information was stratified in 10 min sections with respect to time of arrival and the analysis requested. This allowed the determination of the peak demand and the corresponding analytical spectrum. The instrument capacity was chosen so that it amounted to 3 times the peak demand, which allowed the system to function without a bottleneck during sample preparation or analysis even if an instrument broke down or needed maintenance. We favored multiple, midsize units (centrifuges, analyzers) that provided flexible, parallel processing of the samples in primary tubes.

The organization was checked by determining the turnaround time, i.e., the time from arrival of the samples and requests to the laboratory reception desk up to the time the validated results were received in the ward. This check was performed 1 week at a time, 4 times over a 15 month period, 3 years after initiating the system. The total turnaround time is composed of the analytical time (from specimen reception to results appearing on screen of analyzer) and validation time (from results on screen to printout in the ward).

Sample and Information Flow in the Hospital

On arrival, the patient's information is registered on a BHIS terminal in the central patient admission, the emergency units, or, for external referral samples, at the reception desk of the laboratories. A patient identification (ID) number is created by BHIS and linked to a permanent ID. Bar code labels of the patient ID and alphanumeric name labels are printed (or can be

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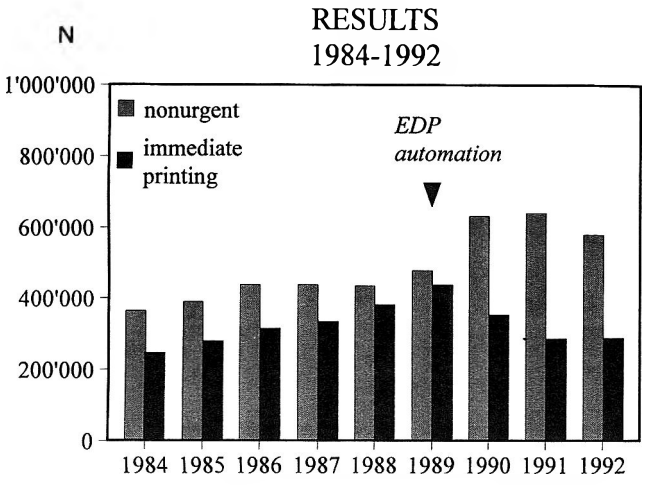

Figure 1. Annual statistics showing the increasing demand and the drop of "stat" (forced impression) requests after reorganization. The number of results requested are shown.

printed in any ward when needed). The request forms (one per each type of specimen) are readable by optomechanical scanners. Preassigned bar code labels are attached for identifying the request number. These labels are put on the sample tubes when collecting the specimen. The patient ID bar code is put on the request form and links the request number to the patient ID.

Sample tubes (Monovette, Sarstedt, Nümbrecht, Germany) with the request identification bar code and a full name sticker are sent in plastic bags with the request forms to the reception desk of the central laboratories by 2 pneumatic transport systems or by transport personnel. The specimen and request form are checked for adequacy. The request sheet is read into the laboratory computer system, which then obtains additional information concerning the patient from the BHIS and connects it to the request. If the request sheets show only demands concerning the most frequent analyses and no special demands, the sheets are stored at the desk; the sample tube (without request form) is handed to the laboratory for centrifugation, sample inspection, and analysis. There is no splitting of sample. Bi-directional analyzers with positive sample identification will read the bar code on the primary tube, which avoids sample confusion, and perform the analyses requested as stored in the Swisslab computer system. Ninety-two percent of the sample load is processed this way.

Request forms for less-frequent analyses (e.g., drug monitoring, amino acid analysis, gas chromatography/mass spectrometry of organic acids) or that contain remarks accompany the primary tube to the analytical laboratory. Thus, a request sheet alerts the technicians. Additional information can be added to the request on the terminals.

Work lists are created before analysis only for those analyses that are not obtained by bi-directional link with positive sample identification (e.g., analyzer COBAS FARA, Roche, TDx, Abbott).

After analysis, the results appear on the screen (printable if needed) together with the preceding results of the patient and the data. This combined information allows technical validation and a delta-check that takes into account the elapsed time since the previous result.

Technically validated results are transferred through the laboratory computer system to the BHIS. If the results exceed predetermined limits, they will appear in a plausibility control list that is validated twice daily by the laboratory staff after additional information has been obtained from the physician, if needed.

Wards have the option of demanding a forced printout of the results. In this instance, results will be printed on a laser printer (85 in the hospital wards) as soon as they are available. In any case, BHIS scans the database at fixed hours preset by the medical services and adapted to the local clinical organization; cumulative result sheets (actual results up to 6 previous results and reference range by age and sex) are transferred into an electronic mailbox of the corresponding ward if any data of the patient have been changed since the last output. The content of the mailbox is printed on a simple macro command at the ward terminal. Restricting the output to the terminal screens proved to be impractical, frustrating, and time consuming in a pilot experiment.

Results and Discussion

The laboratory organization, which does not distinguish between a stat or routine analysis, allows a fluent sample throughput without additional work load for sorting. Because analytical capacity is no longer a problem with modern analyzers, we consider the sorting to be useless with a few exceptions (e.g., ionized calcium after major transfusions of citrated blood). Analyses needed within minutes are obtained in the patient units (e.g., blood gases, glucose stix, and potassium after cardiac interventions). The reorganization performed in 1989 (Figure 1) led to a drop of stat demands, from 48% to 31–34% (forced impression), despite a steady shortening of mean hospitalization (actually 11 days). The drop of stat demands is even more impressive if one considers certain wards that thoroughly understood the system ("stat" demands in neonatal intensive care dropped from 81 to 18%). Recent simulation programs support our practical experience; parallel processing of the work flow is more efficient than batch processing after sample splitting (1).

As shown in Figure 2, the total turnaround time can be kept below 1 h, irrespective of the type of request (median time for analysis, 29–48 min; median time for validation of the results and transfer, 14–21 min). The transfer time from the Swisslab through BHIS to laser printers in the wards is not an important factor of delay (<3 minutes). Samples that are highly pathological and have to be repeated and diluted, or samples that need special treatment (lipidaemic or icteric samples) lead to further delays. The turnaround time depends on the work load, which in our setup is greatest in the morning and when less personnel are available (for instance, delays are longer for validation during lunchtime). The work load further depends on the day of

the week (about 1400 more requests in clinical chemistry are made on Monday than on Sunday).

Further improvements are expected if computerization is not limited to the Laboratory Information Management System or to laboratory management and quality control software but extends to the hospital as a whole system. This extension would require the integration and adaptation of more functions to the information needs of each sector. Therefore, tools operating in an open architecture, preferably with object-oriented language, should allow integration of laboratory requests (on-line) and results into the corresponding agendas, without transcription from or into the medical and nursing file of the patient. In exchange, actual medication, clinical findings, differential diagnosis, and previous diagnoses should be available to the laboratory for the plausibility control and for suggesting diagnostic approaches. Updated information on laboratory performance (e.g., precision of internal quality control and expected turnaround time) should help to tailor the demand to the clinical needs.

Additional modules are also planned for including the financing, inventory, maintenance, and ordering of consumables into the system by linking the laboratory system on the hospital network to other supportive units.

The vulnerability of such a system should be taken into account by duplicating vital parts of the network and instrument connections and by having prepared alternative solutions to circumvent down-times of the BHIS; "break-down" identification bar code labels are prepared in the wards. Confidentiality of the data has to be warranted as well, but procedures must be kept time efficient and user friendly.

In our experience, an increase in communication contributes to a good working climate, fewer harassing phone calls,

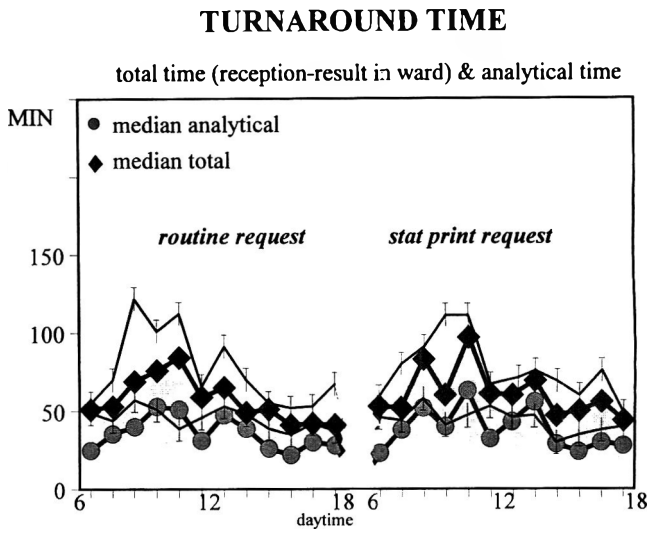

Figure 2. Median values and corresponding ranges of total turnaround time and analytical time (for definition, see text) with respect to time of day for requests received on reception and without forced printing (routine) and for those with immediate printing.

and more personnel satisfaction. Therefore, automation and computerization reduces repetitive tasks and allows more individual input into the work.

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AOAC SYMPOSIUM: AUTOMATED GOOD LABORATORY PRACTICE

Development of Data Sets for the Validation of Analytical Instrumentation

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Analytical chemistry makes use of a wide range of basic statistical operations, including means; standard deviations; significance tests based on assumed distributions; and linear, polynomial, and multivariate regression. The effects of limited numerical precision, poor choice of algorithm, and extreme dynamic range on these common statistical operations are discussed. The effects of incorrect choice of algorithm on calculations of basic statistical parameters and calibration lines are illustrated by examples. Some approaches to validation of such software are considered. The preparation of reference data sets for testing statistical software is discussed. The use of 'null space' methods for producing reference data sets is described, and an example is given. These data sets have well-characterized properties and can be used to test the accuracy of basic statistical procedures. Specific properties that are controlled include the numerical precision required to represent the sets exactly and the analytically correct answers. A further property of some of the data sets under development is the predictability of the deviation from the expected results resulting from poor choice of algorithm.

Over the last 10–15 years, computer control of instrumentation has moved from the exception, typically available only on the most expensive instruments, to the rule. It is almost impossible to buy an analytical instrument without embedded microprocessor control, and the price of personal computers has dropped so far that workstations are available for midrange instruments. Today, computer control may extend through calibration, data acquisition, display, processing, recalculation, and reporting and may even carry out some of the interpretation of results.

The analyst has gained a great deal from these developments. More data are obtained in less time, effective and rapid tools allow better use of the data to solve more complex problems, and the chemist can concentrate more on the chemistry than on the arithmetic. Unfortunately, the data processing steps, which are frequently hidden from the analyst, may represent a break in the analyst's control over the measurement process. Therefore, traceability from the measurement to the result becomes clouded. In particular, it is very difficult to show that the data processing is performing correctly.

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Statistical operations are fundamental to analytical chemistry and cover, for example, determination of means, standard deviations, quality control charting, and calibration using regression methods. In this paper, we illustrate some of the problems inherent in computer data processing and describe an approach to testing computer software that performs statistical operations. We show that it is possible to prepare data sets capable of testing the degree of departure from ideal operation.

Discussion

We will discuss 3 main problem types: problems relating to computer precision and dynamic range, poor choice of algorithm, and invalid use of procedures. A fourth type of problem, incorrect coding, will be considered briefly without illustration.

Computer precision and dynamic range are closely related. Precision is the number of significant figures the computer can accurately represent; dynamic range, for our purposes, is the ratio between the range of values covered and the highest value within a single calculation. (Dynamic range is really a property of the problem at hand, but it is the computer's ability to cope that is the issue here.) For most applications, computers use floating point arithmetic; this effectively reduces most dynamic range problems to precision problems.

Standard Deviation

The repeatability standard deviation of a 4-figure balance was required as part of a reference material uncertainty estimate. Successive weighings of a nominal 50 g weight produced a set of results comparable to the list in Table 1.

Because there were so few figures, a pocket calculator with statistics functions was used to calculate the standard deviation. The calculator was a well-known brand operating to 8 significant figures, so the 6-figure precision looked unlikely to cause

Table 1. Replicate weighing data

50.0001	50.0001	50.0002
50.0001	50.0000	50.0001
50.0000	50.0000	50.0000
50.0000	50.0001	50.0001
49.9999	50.0001	50.0001

problems. The value displayed, however, was identically zero, rather than approximately 7×10^{-5} as expected. This anomalous result prompted further tests on a range of software and hardware (Table 2). Agreement between the results was poor; values ranged from 0 to 7.298×10^{-5}.

The reason for the seriously anomalous performance of the first calculator lies in the precision required by the calculation. In this instance, even though the microprocessor could accurately represent the data, it failed to represent the intermediate values in the calculation. To provide an updated standard deviation without storing each data item, the calculator used a sequential algorithm and stored only the following values: n (number of data items), $\bar{x}$, Σx, and Σx^2. The sample standard deviation was calculated, on demand, by using the following equation:

$$\sigma = \sqrt{\frac{\sum x^2 - \frac{(\sum x)^2}{n}}{n-1}}$$

By comparison, other software used a simultaneous calculation across all data points held separately in memory and calculated the standard deviation as follows:

$$\sigma = \sqrt{\frac{\sum (x_i - \bar{x})^2}{n-1}}$$

Table 3 shows the precision requirements of the 2 algorithms for the data set in Table 1. Note that the sequential approach requires 12-figure precision in the intermediate value Σx^2. Neither of the quadratic terms can be represented by 9-digit arithmetic in this case; truncation leaves the 2 terms equal in the calculation.

Table 2. Standard deviations calculated for data in Table 1

System	SD × 10^5
Calculator 1	0.00
Calculator 2	7.298
Calculator 3	7.182
LIMS system	7.1
Spreadsheet	7.180
BASIC (single)[a]	7.212
BASIC (double)[a]	7.174
PC Calculator	5

[a] Microsoft GW-BASIC running on an Olivetti M300 equipped with floating-point coprocessor, using single and double precision arithmetic, respectively, and using the 'simultaneous' calculation described in the main text.

Table 3. Precision requirements for intermediate values in the calculation of standard deviation (in significant figures)

Value	Simultaneous	Sequential
n	2	2
$\bar{x}$	6	6
$\sum x$	—	7
$(\sum x)^2$	—	12
$\sum (x^2)$	—	12
$\sum (x_i - \bar{x})^2$	3	—

The systems tested were proprietary systems and were, apparently, within their normal range of operations and functioning according to their specification. Nonetheless, many of the answers were inaccurate or badly incorrect.

Calibration

In this laboratory, we use an atomic absorption instrument with an autocalibration feature. It takes information on 3 or more reference samples, requests a number of replicates of each reference, and calculates calibration parameters that are stored internally. Thereafter, results are given directly in concentration units. Because this instrument is old, the output is on dot-matrix thermal tape. During the calibration, the absorbance values, input concentrations, and an RMS error for the calibration are printed. Table 4 gives a simulated data set formed from original data, adjusted as noted to highlight the issue discussed,

Table 4. Atomic absorption calibration data and regression results[a]

Concn, mg/L	Absorbance	Concn, mg/L	Absorbance
5.0	0.091	10.0	0.232
5.0	0.092	10.0	0.234
5.0	0.090	15.0	0.258
5.0	0.092	15.0	0.255
5.0	0.091	15.0	0.255
10.0	0.232	15.0	0.258
10.0	0.227	15.0	0.260
10.0	0.227		

Regression: Std error of Y	0.0022876
r^2	0.9992099
No. of observations	15

[a] The table shows a calibration set obtained for a single element determination, with the absorbance for all 10 mg/L replicates increased by 10%. The table of residuals gives the information provided by the original instrument, recalculated for the simulated data set in the table.

together with the regression results. The results show a very good RMS error, which indicates great confidence in the calibration. Overall results are apparently excellent. However, closer study is essential. There are actually only 3 reference samples, though each was run 5 times. Close examination of the data also shows that the absorbance/concentration relationship is not linear, and there is some reason to wonder whether the concentrations of the calibration set are correct.

What the software has done in this case is fit an arbitrary curve through the data, rather than a straight line. Because we need 3 parameters to define the curve, and we have only 3 independent values, the curve fits the data exactly, and the residual error results solely from the run-to-run repeatability of the instrument. Figure 1 shows the curve obtained from the values in Table 4 by using a standard curve-fitting package. It is immediately clear that the input data are unreasonable; either the detection is far from linear, or at least one of the reference concentrations was entered incorrectly.

The issue here is 3-fold. First, the software carried out a regression in such a way that there was no information available as to the validity of the data set. Second, there was no immediate way of telling from the output that anything was wrong; in fact, the RMS value suggested that all was well. Third, there was no indication anywhere of what the assumed curve form was; that is, not only was the algorithm in use inappropriate except in a very limited range of cases (mathematically speaking), but there was no way to predict the limitations of the software.

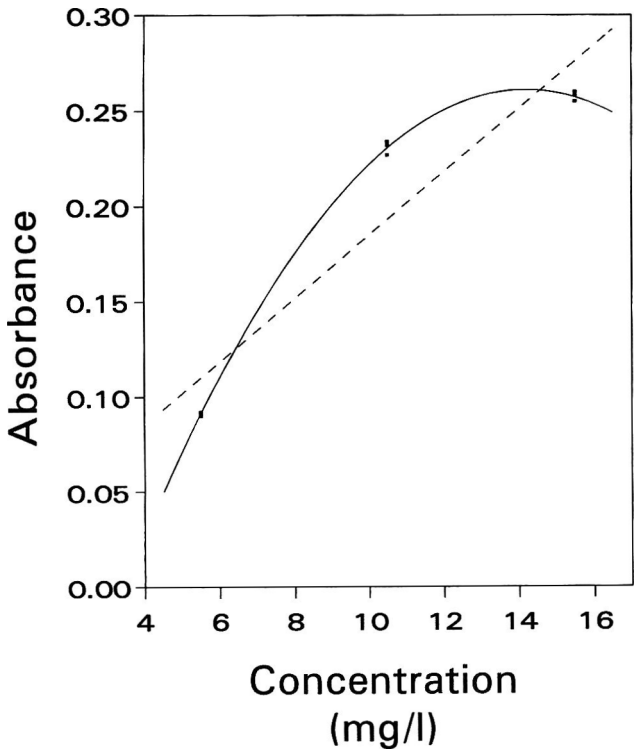

Figure 1. Incorrect use of curvilinear regression; data points (■) are from Table 4: (———) best fit parabola, (– – –) best linear calibration.

Software Validation

There are clearly many potential sources of error that can deliver an incorrect analytical result. If there are chemical sources of error, then independent accreditation, use of certified reference materials, proficiency testing scheme participation, and use of validated methods are to be recommended. However, validation of software against specification is extremely difficult. The most general approach is the 'formal methods' approach pursued by several computer science research groups. Formal methods involve specification in terms that allow mathematical proof of function, and further allow direct conversion of specification to program code. The theoretical result is that the program is mathematically certain to perform correctly. So far, however, formal methods have some way to go before reaching the world of commercial software development.

The next best approach to software validation is to test software against known inputs and outputs. This compares to the use of Reference Materials in chemical method testing. Data sets for such testing should have well-understood properties. In particular, finite numerical precision should represent the data exactly; solutions for each set should be known exactly; the sets should be transferable across different hardware platforms; use of the sets should show the extent of deviation from the ideal; and the behavior of different algorithms on a set should be known. We now show how such sets can be generated in the case of regression analysis.

Data Set Generation Using Null-Space Methods

The preparation of reference data sets for linear regression provides an example of the principles involved. Figure 2 shows the situation. x and y_0 form a set of data pairs falling exactly on

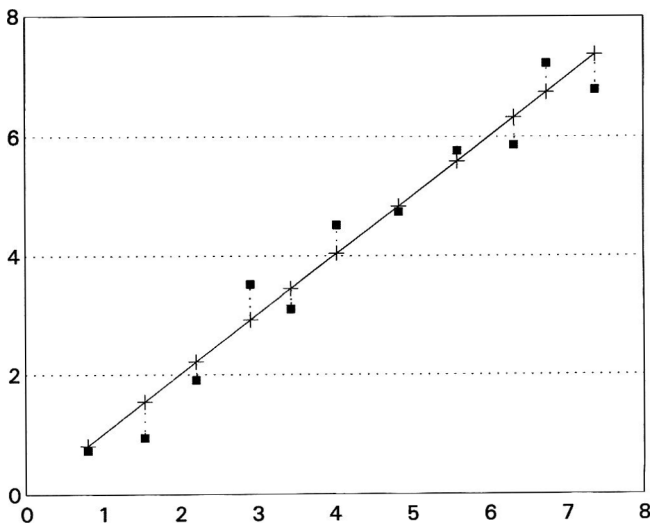

Figure 2. Construction of perturbed linear regression test data; (———) equation for line: $y = u_1 + u_2x$; (+) points (x_i, y_i) falling on the line; and (■) perturbed points $(x_i, y'_i) = (x_i, y_i + c_i)$.

a straight line $y = u_1 + u_2 \cdot x$ characterised by the parameters u. In matrix form:

$$A \cdot u = y_0$$

where A is the 'observation matrix' given by the following:

$$A = \begin{bmatrix} 1 & x_1 \\ 1 & x_2 \\ \dots & \dots \\ 1 & x_n \end{bmatrix}$$

and $u = [u_1, u_2]^T$ is the vector of parameters. Note that the model is readily extended to any number of independent linear coefficients in u by increasing the number of columns in A and rows in u.

Now, given a set of values x, y', where y' is a set of experimental observations, the aim of linear regression is to find u such that some function of u, A and y' is minimized. In least-squares regression, the function is simply the sum of the squares of the residuals $\Sigma(y'_i - y_{0_i})^2$. A number of algorithms have been developed to carry out linear regression (1–5).

To test software written to carry out this task, a number of possible approaches can be envisaged. In the simplest, a set of (x, y) pairs falling exactly on a known straight line (u known) could be generated. This provides a direct test of whether the software delivers identically correct answers in ideal situations, but fails to show how nonideal data are dealt with. Further, ideal data will often cause problems when error estimates are also determined, because errors should be very small. This in turn may prevent termination of iterative fitting methods, which often rely on convergence of the error estimate as a termination condition. Alternatively, a common approach in software testing is to generate such a data set and randomly perturb it, using, for example, a random number generator. This gives a test of software function, but, because the perturbation is not characterized accurately, the line of best fit is not known, and the test will not show how close the software comes to a best fit.

In the approach we have taken, these simpler approaches are combined. A data set is generated from an analytically known line such that the best fit to the perturbed data set is identical to the original line. To see how this is possible, note that the best fit value of u by the least-squares criterion is the solution to the normal equations:

$$A^T \cdot A \cdot u = A^T \cdot y$$

If c exists such that:

$$A^T c = 0 \qquad [1]$$

it follows that u will also be the solution to:

$$A^T \cdot A \cdot u = A^T \cdot (y + c)$$

Given u, therefore, we need to seek c as a solution to equation [1]. Two points should be noted. First, in this instance, c is a function only of the observation matrix, which is independent of y. Second, c is, for this purpose, under determined. For n equations in m unknowns, the set of vectors is a vector space of dimension $n - m$. There are, therefore, $n - m$ degrees of freedom in specifying a vector c satisfying equation [1]. The vector space referred to (satisfying [1]) is known as the null space of A_T; this gives rise to the term 'null space method,' which we use to refer to this method of generating test data sets. The number of possible solutions allows us to impose additional constraints on c. Specifically, given integer A, we can find integer c, allowing direct control of the numeric precision. Table 5

Table 5. Reference data set for linear regression analysis[a]

X	Y	Residual
−7.3970	−7.4410	1.1200
−6.1650	−7.9450	−0.7840
4.7470	8.3190	3.0800
−5.5490	−6.5730	−0.1120
−0.3570	−5.9930	−5.4320
−7.3970	−12.8170	−4.2560
−6.4290	−1.6930	5.7680
0.3470	−0.7690	−1.0080
8.7950	9.6150	−0.2240
2.9870	4.5270	1.2880
6.0670	9.7070	2.9680
−6.5170	−5.3770	2.1840
9.2350	16.7230	6.3840
9.2350	4.3470	−5.9920
−1.3250	0.6910	2.3520
−2.3810	−7.1730	−4.3120
8.0030	5.9150	−3.0240

[a] The table shows an example of a 17-point reference data set. The residuals are $(y_i - y_{calc})$ for the line of best fit.

Table 6. Effect of X translation on linear-regression residuals[a]

T	A1	A2	A3	A4
10	2.60×10^3	2.91×10^3	1.78×10^4	1.41×10^5
10×10^2	8.31×10^3	8.43×10^3	6.29×10^3	1.03×10^5
10×10^4	2.42×10^5	2.42×10^5	1.88×10^5	1.13×10^8
10×10^6	1.88×10^7	1.88×10^7	3.18×10^7	3.2×10^{12}
10×10^8	1.05×10^9	1.05×10^9	4.43×10^9	2.1×10^{16}
10×10^{10}	2.4×10^{11}	2.4×10^{11}	1.8×10^{19}	1.9×10^{19}
10×10^{12}	4.2×10^{12}	4.2×10^{12}	1.8×10^{19}	1.8×10^{19}

[a] Figures given are the norm of the difference between calculated residual vector and reference residual vector, with a translation of T along X ($x_i \rightarrow x_i + T$) for each of 4 algorithms applied to a 31-point reference data set. A1 uses a stable formulation in which the data mean is subtracted before summing and squaring; A2 uses an orthogonal factorization on mean-centered data; A3 uses an orthogonal factorization without mean-centering, and A4 uses a 'single pass' algorithm without precalculation of means.

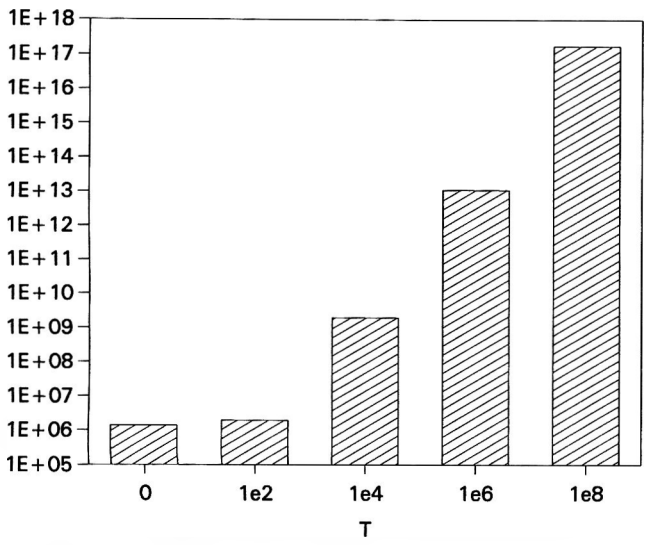

Figure 3. Variation of normalized residual error with x-axis translation. Note the logarithmic vertical scale.

shows a data set prepared in this way. Note that all figures given in the table are exact; no rounding or truncation has been performed.

Finally, note that c is the vector of residuals for the line of best fit. For any translation of the data, the best fit line will show the same set of residuals c. Compare this to the variation of u on adding a scalar to x or to y. This makes the difference between c (which is analytically known) and the residuals $\hat{c}$ calculated by software under test an excellent check on the accuracy of the software. Further, this allows generation of 'ill-conditioned' data by simple translation of the basic test set along the X axis. Table 6 shows the effects of such a translation on the residuals for different linear least-square algorithms.

Finally, Figure 3 shows the variation of $\Sigma(c_i - \hat{c}_i)^2$ with translation for the data set of Table 6 for a commercial software package. Direct comparison of these data with that in Table 6 shows that the package compares with the worst performing algorithms tested. The commercial package, therefore, probably implements linear regression via the normal equations without any attempt to normalize or re-center the data and probably uses double precision arithmetic. This particular software package would be expected to give increasingly inaccurate results as the ratio of the data range to its mean decreased, but for reasonable data (range/mean ratio ≤100), no serious errors would appear in the first 3–4 figures of the solution.

Conclusion

We have shown that finite numerical precision, aggravated by poor choice of algorithm, can cause significant errors in software designed to process statistical data, including that obtained in analytical chemistry. Data sets with well-known characteristics can be developed to test such software, revealing the behavior of the underlying algorithms. This information can be used to establish the scope of acceptable operation for software and provide a useful tool for validation in practical applications.

Note: Reference data were prepared on a Digital Equipment Corporation VAX 6300 series computer running VMS. Pro-Matlab software (6) was used to carry out the calculations.

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AOAC SYMPOSIUM: AUTOMATED GOOD LABORATORY PRACTICE

Information Technology/Laboratory Information Management Systems: Problems of Regulatory Compliance

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Despite the attention paid to the regulatory requirements for computer systems, recent inspections continue to report basic failings in the standards of validation and documentation. This paper describes the need for validation of vendor-supplied software such as Laboratory Information Management Systems and the procedures necessary for control of the production phase in a regulated environment. Whereas these requirements may be considered "good computing practices," their adherence remains a management challenge at odds with pressures of cost, business need, and time.

This paper focuses on those measures necessary to manage, implement, and operate a computer system whose data are used for regulatory purposes. After a brief review of the good laboratory practice (GLP) principles that apply to computer systems, these ground rules are applied to the development, implementation and production phases of the computer system life cycle, giving an overview of the essential elements that lead to regulatory compliance.

GLP Regulations

GLP regulations were first introduced almost fifteen years ago by the U.S. Food and Drug Administration (FDA) following evidence that a number of reports from toxicology studies had contained both inaccuracies and deficiencies. These regulations were the forerunners of similar principles and guidelines for GLP worldwide. As an example of the concerns raised almost 10 years ago, an FDA inspection showed a lack of evidence, i.e., documentation for validation of the computer system, a lack of standard operating procedures (SOPs) and no evidence of inspection by a quality assurance (QA) unit. This led to the conclusion that the quality of data processed was, at best, uncertain.

With the number of meetings, papers and courses dedicated to the regulatory requirements applying to computer systems, one might conclude that these concerns should have been addressed by now. However, this is not the case, as recent reports of FDA inspections show. These points were taken from 6 FDA 483 reports of inspections conducted from June to December 1992 at major U.S. pharmaceutical companies and contract laboratories. Computer systems were not considered validated for the following reasons: incomplete or ambiguous specification, validation not planned, test results not retained, not tested with invalid data or boundary conditions, validation environment inappropriate, and no standards or procedures defined. In summary, exactly the same conclusions were reached in these recent FDA 483 reports as in the earlier inspections: inadequate documentation, insufficient validation, and poor quality of validation all pointed to a lack of management control.

When the first GLP regulations were introduced, few companies had computer systems for data capture and processing. Indeed, the first U.K. GLP regulations in 1986 hardly mention the terms 'computer' or 'software,' with only 4 such references in 18 pages of small type.

As computerization spread, GLP had to adapt or be interpreted. Revisions of the GLP regulations (1) have given increasing importance to computer systems and a number of GLP/computer system references and guidelines were published (2–4).

The fundamental purpose of GLP is to assure the quality and integrity of the data submitted in support of regulated products. GLP application to computer systems yields a familiar set of principles that cover the following: standard operating procedures, personnel, training, security, raw data, documentation, equipment, involvement of a QA unit, and archive.

Any computer system operated in a GLP environment must provide evidence that demonstrates that each of these principles has been considered and is under control. Examples of typical evidence would include policies, procedures, standards, specifications, user guides, training records, and curriculum vitae.

Development of a Laboratory Information Management System

Does a purchased laboratory information management system (LIMS) require any development?

Almost invariably, packaged software such as LIMS is unlikely to provide a perfect match with an organization's requirements. The purchaser has the option of either creating new working practices that comply with the new computer system or modifying the software to meet the actual requirements.

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LIMS is a system that is commonly considered by its purchaser to be 'load and go.' Whereas for components such as a chromatography sub-module this may be largely true, the data management needs of each user company are sufficiently different that some additional development is usually necessary. LIMS systems should be seen more as tool kits that need assembly from a number of preformed components to make the whole rather than complete within themselves. With the increased functionality in modern chromatography systems such as Perkin-Elmer's "Access * Chrom," the traditional data processing role of LIMS is migrating toward the sub-module, making the definition of LIMS even more uncertain and specific to each user.

Thus, with a LIMS system, any development is most likely to take the form of tailoring or adding pieces to the edges of the standard system. Like any software development for GLP purposes, this requires full documentation (i.e., specifications) and formal testing prior to implementation. In most instances, this should not be an onerous task; the development itself is an enhancement to the purchased LIMS system and similarly the user specification should make extensive reference to the supplied LIMS documentation. Such tailoring could be undertaken by the vendor as part of the purchase contract, but regardless of who does it, it must be documented completely.

Validation

In the GLP environment, computers may be considered to be just like any other piece of laboratory equipment and thus must be demonstrably functioning in the proper manner. This proving phase of systems development is validation, which is a step in the System Development Life Cycle of Validation. The conduct has been heavily criticized in regulatory inspections.

Chamberlain (5) suggested the following 5 'simple principles' for validation of any computer system: The user is responsible for the validation of the computer system; there must be a validation protocol for the system; the protocol must be executed and the results documented, archived, and signed off; there will be SOPs; and there must be evidence that some QA group is auditing the computer system. A QA unit does not conduct validation but only checks that the validation has been performed to a satisfactory standard.

Typically, a validation plan comprises the design, execution and recording of the results from a series of tests with suitable data that demonstrate that the system performs the functions it is intended to perform and does so satisfactorily. Parallel testing alone will not satisfy the validation requirement. Although parallel tests can provide useful data, they are not designed to test boundary conditions, responses to erroneous data, or infrequently used options, which are areas where computer systems may prove deficient.

User firms are ultimately responsible for ensuring the adequacy of vendor-supplied software. In the experience of one FDA inspector, "Many firms assume, without having documental evidence to support their assumptions, that vendors have validated their programs."

In every case, user responsibility for validation means that some form of user acceptance testing using the purchaser's hardware is essential, i.e., on the configuration and setting closest to where production use will occur. If the vendor provides appropriate test data and documentation for validation, this process may be foreshortened, but not omitted, for packaged software.

Equally, it is not unreasonable to ask the vendor to help the customer validate the system by providing a system description, summary of the vendor's test procedures and even sample validation plans. However, the basic principle remains that a vendor cannot provide a validated system for the customer to run without further proving. Once in production it is the user who is responsible for the data that are collected and processed, not the vendor.

The FDA-sponsored 'Red Apple' Consensus Workshop (2) suggested that a validation plan should contain the following sections: purpose; test environment; assumptions, exclusions and limitations; responsibility/authority; data sets; test description; expected results; criteria for acceptance; error resolution; and documentation. In practice, we have found this to be a sound framework with the detail required to complete the plan being dependent upon the size and nature of the system and whether the application is an in-house development or a vendor supplied package.

To satisfy a GLP inspection, all validation documentation needs to be written, appropriate and clear, to have its accuracy verified and finally to be approved. Therefore, formal approval of the validation plan is required prior to execution by the project team, who are typically representatives from the user department, computer department and QA unit. Once the plan has been executed, results should be reviewed by the project team who should approve the completed plan and recommend implementation for production use. The plan, its results, and approvals should be retained throughout the production life of the system.

Even though a comprehensive validation cannot raise a poorly designed or unprofessionally programmed system to a GLP standard, many systems in production today are let down by inadequate or non-existent evidence of validation. One U.S. Good Manufacturing Practice (GMP) inspector, Ronald Tetziaff, considers that the planning documentation is one of the most reliable predictors of GMP problems (6). According to Tetziaff, "If a firm does not have a formal written validation plan, then it is impossible for the system to be in a state of validation. Conversely, a well-written plan reflects favorably on the overall quality of a firm's program."

Change Control

In practice, we should expect that the execution of the validation plan will not be perfect. In validation, errors will be found that are usually attributable to software faults. Unless an organization adopts a policy of restarting validation every time an error is found, a policy which is impractical because of cost and time constraints, then management of change through a formal procedure is essential.

If a company has adopted quality development procedures, then a change control procedure will be present in earlier phases to monitor and manage changes to requirements, specifications, and early versions of the software. However, although professionally commendable (and recommended) throughout the development process, it is during the validation and production stages that change control is mandatory in a GLP environment.

Errors discovered during validation that lead to system changes should be documented and this documentation will form part of the total validation evidence. Assessment of the impact of the change is important so that staff responsible for validation may determine which parts of the validation plan need to be repeated once the software has been changed. In most cases, it is not satisfactory simply to repeat the single test that failed; related tests should also be re-executed.

The other key element of note in a change control procedure is authorization to implement the revised software. This is most important when the change is to the system in production, where any software implemented without complete testing could have a major impact on the data collected from a live study. It is imperative that changes to packaged software are also subject to the same regime. Vendors need to be made aware of the need to comply with an organization's change control procedure, a requirement that can become an issue where the vendor wishes to apply changes remotely via a dial-up line. All evidence of changes to the system should be retained.

Production Use

Following approval of the validation plan and its results, the new system is implemented for production use. During this lengthy phase, the emphasis of GLP compliance is upon management control of the computer system. The U.K. Department of Health advisory leaflet on computer systems (3) states that the objectives of a computer system inspection are to ensure the following: Any computer systems that are used for regulatory submissions are suitable for the intended purposes, procedures exist to adequately control and maintain the systems, and systems are operated in a way that is compliant with GLP principles (3).

The first point should be satisfied by the validation process described above. Satisfaction of the other 2 requirements is dependent upon the personnel involved with the system - particularly its users - and the procedures in place around the computer system in production.

Personnel are critical in the operation of a GLP compliant computer system. For example, the integrity of raw data demonstrated during validation can only be maintained to the satisfaction of the QA unit if users are trained in all operational aspects of the system, including the action to be taken in case of system failure. The number of users who know what to do if there is a power failure or a hardware fault and the manner in which data collection will proceed once the system becomes operational should be addressed.

Documentary evidence necessary to demonstrate control includes policies, SOPs, operational records, maintenance agreements, and personnel data. The key documents and records required during the production phase are as follows: hardware configuration, software register (operating system, utilities, and application), problem/fault log, hardware/software change control records, maintenance agreements and records, system personnel authorization/responsibilities, staff training and personnel records, application/system use guide, data backup and restore records, data archive records, and ongoing validation.

One issue in the production use of the system is that of ongoing validation or re-validation. This is the re-execution of the validation plan prepared prior to implementation to prove that the system continues to meets its specification and that there has not been any drift caused by accumulated changes, each of which was only tested individually.

The U.S. Environmental Protection Agency (EPA) made a recommendation in its Good Automated Laboratory Practice guidelines (4) that systems should be re-tested every 24 months.

Perhaps with vendor-supplied software this issue will be less important as suppliers tend to offer new releases every 18 months or so. These will need validating in their own right prior to implementation, and if done in a timely manner, the EPA's 24-month limit will never be reached.

Revalidation is a costly demand both in terms of time and labor, even if some form of keystroke capture and replay software is used. If the system security is adequate and a quality change control procedure is operated, then will re-validation add significantly to the confidence placed on our data?

Summary

It may surprise some practitioners that, despite its prominence in the last 4 years, the term 'validation' is not found in the regulations or the UK Advisory Leaflet on Computer Systems (3).

This reference more appropriately addresses the balance between development and implementation, on the one hand, and production use on the other. Greater emphasis is placed on operational use, where the raw data will be collected and/or processed. One DoH view recently has been that only if problems were found with actual study data would there be a case for examination of the development and validation procedures and documentary evidence.

For a study-based inspection, this is a reasoned approach but does not give management or an inspector confidence in the overall regulatory compliance of the computer systems. The more common facility inspections will tend to begin with the general management of the computer facility, such as its configuration, staffing, maintenance arrangements, security, and back-up, and then move into specific applications typically starting with the validation process. Evidence in the form of SOPs, formal project approvals, system documentation, and operational records will be vital to demonstrate the professional management of the system.

Even though quality development, validation, and implementation procedures are the foundations of a quality production system, it is the production phase that is most critical to GLP. Experience in GLP QA/computing issues is still limited, but I contend that to date too much emphasis has been placed on validation, and insufficient attention has been paid to managing the computer system in production. The GLP goal is to assure the quality and integrity of data submitted in support of the safety of regulated products. I predict a growing interest in operational procedures and records to complement and balance the requirements of the validation process.

Most of this is not new to the computing industry; computing professionals should already consider these demands 'good computing practices.'

However, the computing practitioner is renowned for knowing what to do but ignoring his own advice. Lack of documentation or scanty documentation prepared after implementation are classic examples in this regard. The ubiquitous PC user is even less likely to prepare documentation. Pressures of commercial expediency/time constraints versus quality/GLP demands are seen in opposition in many laboratories. This is why, in conclusion, I consider good computing practice to be the challenge that faces all those responsible for computer systems.

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AOAC SYMPOSIUM: AUTOMATED GOOD LABORATORY PRACTICE

Automation and Quality in Analytical Laboratories

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After a brief introduction to the generic aspects of automation in analytical laboratories, the different approaches to quality in analytical chemistry are presented and discussed to establish the following different facets emerging from the combination of quality and automation: automated analytical control of quality of products and systems; quality control of automated chemical analysis; and improvement of capital (accuracy and representativeness), basic (sensitivity, precision, and selectivity), and complementary (rapidity, cost, and personnel factors) analytical features. Several examples are presented to demonstrate the importance of this marriage of convenience in present and future analytical chemistry.

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Automation has become a trend in analytical laboratories at the same time that quality has become a crucial demand in social, economic, technical, and scientific fields. Even though quality has been a continuous objective in analytical chemistry for years, the present implementation of quality assurance principles in analytical laboratories and the dramatic developments in automated instrumentation and methodologies make the marriage between automation and quality absolutely necessary in today's analytical laboratories. We address the joint aspects of both concepts; mainly, the automated analytical control of external quality, quality control of automated analysis, and how the analytical quality can be improved through automation.

Automation in Analytical Chemistry

Automation involves the partial or full replacement of human participation in a given process. One should distinguish here between human labor, perception, and intelligence, which

can be replaced with apparatus, instruments, and feed-back systems, respectively. Automation entails the use of systems (apparatus plus instruments) incorporating one or more feedback devices. Therefore, automated systems include both a programmable event controller, a variable controller, and possess decision-making capabilities. The difference between "automated" and "automatic" systems lies in whether or not human intelligence (that is, the ability to do something appropriate under unpredictable conditions) is replaced (1).

There are 3 general approaches to automating the analytical laboratory: (*1*) automation of laboratory management, which entails controlling such things as samples, reagents, apparatus, instruments, raw data, reports, quality systems, and personnel profiles in an automatic manner by using appropriate software; (*2*) automation of analytical laboratory operations by using modular or integral analyzers; and (*3*) overall automation that integrates the previous 2 approaches and is based on 1 of the so-called "Laboratory Information Management Systems" (LIMS).

The main goals of automation of analytical processes can be summarized as follows: minimization of errors resulting from the human factor and reduction of costs and hazards; processing of a large number of samples at a high rate; reduction of the amount of sample and reagent consumed; enhancement of such valuable analytical properties as sensitivity, selectivity, precision, and scope of application relative to manual methods; and implementation of a wide variety of modern analytical methods and techniques. Despite these advantages, one should also be aware of the following risks involved in increased reduction of human participation in laboratory processes: the operator is to a great extent detached from the analytical operations, which results in the occasional loss of valuable (though unpredictable) information; overestimation of the real capabilities of automation can occur; the ease with which results are obtained may foster avoidance of critical discussion; and decreased flexibility can result from the need to adjust to ready-made software.

The development of analytical chemistry has not been too harmonious. The last 2 stages of the analytical process (measuring and transducing of the analytical signal, and data collection and treatment) have reached a state of development that could be considered satisfactory. On the other hand, advances in preliminary operations have run at a much slower pace despite their doubtless, decisive significance to obtaining quality analytical information in a rapid, economic and human/environmentally safe way. This situation is consistent with the degree of automation reached in each of these steps. Chemometrics in the third stage and automated instruments in the second are now commonplace in the analytical laboratory. So far, preliminary operations have scarcely been automated, despite the great interest in reducing human participation in such time consuming, tedious activities that are also the source of major errors and are, occasionally, even hazardous. Automation of the preliminary operations connecting the uncollected, untreated, unmeasured sample with the instrument is a major goal of analytical chemistry on the verge of 21st century (2).

Analytical Quality Concepts

When analytical chemistry and quality are combined, one should distinguished between "external quality" (related to the characteristics of products and/or systems from the public or private body to which the analytical laboratory is answerable), and "internal quality," which in fact makes analytical quality. Internal quality is in turn related to performance of the analytical process and accuracy of the results. Thus, one must distinguish between the following several quality concepts related to analytical chemistry: quality of the external products or system, quality of the analytical process, and quality of the results. In summary, the analytical quality is part of the total or integral quality. Analytical laboratories as services can help in checking and improving the quality of products, systems, and other services.

Quality control is mainly intended to check the characteristic parameters of the analytical work, materials, apparatus, instruments, software, and, ultimately, the analytical results. Quality assessment activities in turn check for quality control. Both are parts of quality assurance.

A relationship exists between analytical quality concept and analytical features. Analytical properties can be classified into 3 groups according to their relative significance: capital (accuracy and representativeness); basic (sensitivity, selectivity, precision, and sampling), which are the foundation of the capital properties; and accessory (rapidity, low cost, and staff safety and comfort). Capital properties directly influence the quality of results, whereas the other 2 types of properties affect the quality of implementation of the analytical process.

The implications of combining quality and automation in the analytical laboratory can be implemented by answering the following questions. How can automated analytical control help in achieving external quality? How can quality control improve on the features of automated analysis? How can automation enhance analytical quality?

These 3 sides of automation and quality are considered in the following parts of this paper when they merge in the laboratory.

Automated Analytical Control of External Quality

Automated analytical quality control as a part of integral or total quality can help in assuring quality of the products, system, and services in a more efficient way when compared to nonautomated analytical approaches (Figure 1). A typical example in this context is process monitoring of industrial, ecological, or production systems (3). There are 5 approaches to increasing the degree of automation. In off-line monitoring, sample and sample transport are performed manually. In at-line systems, sampling is performed manually and samples are rapidly transported to a dedicated instrument (an analyzer installed in the vicinity of the evolving system). In on-line systems, sampling and sample monitoring are completely automated and are built into the analyzer. In-line analyses rely on the use of physical, chemical, or biochemical sensors. Finally, in noninvasive approaches (e.g., use of spectroscopy with optical fibers or ul-

trasounds), no physical contact exists between the sample and probe (or instrument).

Our research team recently developed a fully-automated flow-system developed for the on-line monitoring of 5 quality parameters in the input/output seawater streams at a young fish breeding farm (4). It has been optimized for the wet-chemical photometric sequential determination of ammonium (by using Nessler's reagent mixture) and nitrite (with the Griess reagent mixture), as well as the continuous monitoring of pH, oxygen, and conductivity by using suitable direct in-line sensors. A microcomputer furnished with active and passive interfaces allows one to control the functioning of valves and pumps and to acquire, process, and deliver the results as required at a throughput that depends on fish size. A high rate is needed when fish are at their earliest growth stages.

The automated on-line monitoring to control the preparation of Alka Seltzer tablets (from Miles, Indiana, USA) is another example in this context (5). The manufacturing steps include compression, sealing, cartoning, bundling, packaging into shippers, and transfer to the warehouse. The test areas are hardness and weight testing, assay testing, uniformity control testing, and leakage testing (to assure the aluminum pouches are tight because of the moisture sensitivity of the effervescent system). The main objective is that every test should have been completed by the time the cartons are checked and the material sent to shipping. Dedicated robots will play a major role in this automated approach in the future.

Quality Control of Automated Analysis

The second aspect of the combination of automation and quality in the analytical laboratory is how quality control systems can improve the quality of automated analysis and is briefly discussed here. This is probably the most interesting approach on account of the big changes performed in laboratory work and the need to increase the degree of automation responding to the growing demands for good analytical results obtained in an efficient way. This need was recognized several years ago by the National Institute of Standards and Technology (NIST), which established the industry/government Consortium on Automated Laboratory Systems (CAALS). CAALS took into account that the number of analyses with which industrial laboratories will be confronted is bound to grow by a factor of 3 during the present decade. Developing and applying automated analytical techniques and standard methods for automated laboratory systems, training technical and professional staff, bringing chemical analysis expertise into the automatic operation of the laboratory system, and introducing quality assurance into automated systems are the chief objectives of CAALS (6).

Quality control should minimize or avoid the main risks faced in the automation of the analytical laboratory. The main objective in this respect is to increase the number and variety of checks performed on the overall analytical process, instruments, apparatus, and materials and to circumvent the problems arising from detachment of the human operator from the analytical process. As human participation is reduced (from conventional manual to fully automated systems), the need for quality control increases. This is quite a general rule that must be taken into account as automation is gradually introduced in the analytical laboratory.

As can be seen in Figure 2, quality control can efficiently help in checking and improving the 3 types of analytical properties (capital, basic, and accessory), which influence the quality of results and processes in automated analysis. Several relevant approaches in this context are described below.

Representativeness is a unique feature of the analytical results that can be defined as accordance of the results with the definition of the social and analytical problem concerned, as well as with the bulk sample and aliquots from which the analytical process starts. The only way to control this quality parameter in automated analysis is by applying chemometric-statistical methods. Quality control of accuracy in automated

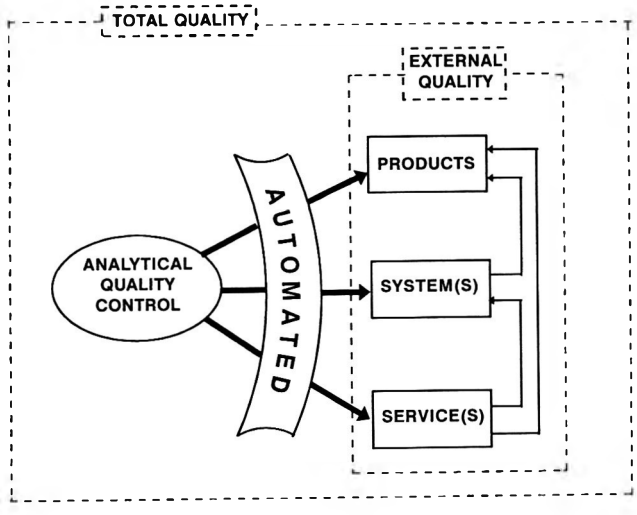

Figure 1. Contribution of automated analytical quality control to total quality.

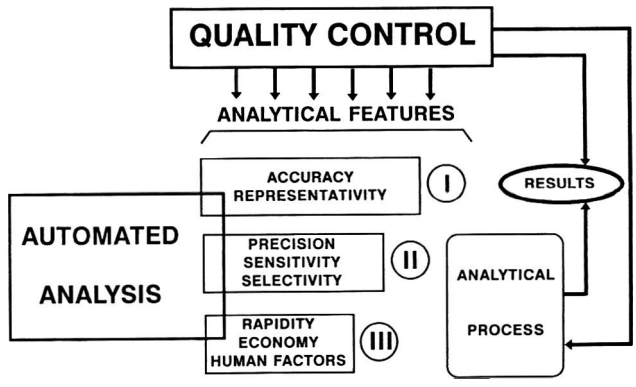

Figure 2. Quality control of automated analytical processes and the corresponding results through the control of capital, basic, and complementary analytical features.

analytical processes (Figure 3) can be implemented by using automation approaches involving reference materials (whether certified or not) and chemical standards to assess the overall analytical process and implement calibration procedures, respectively. Chemometrics plays a major role in dealing with calibration and systematic study of the results (e.g., statistical comparison between the results obtained from CRMs and samples).

Quality of results (accuracy) in automated analyses relies on appropriate calibration, which in turn depends on the use of high quality reference materials and standards. The relationship between automation and calibration is quite contradictory. As a rule, the higher the degree of automation (and simplication or miniaturization) the more difficult the implementation of calibration procedures. Such is the case with the various monitoring approaches; from off-line to non-invasive, the degree of automation increases as the ease of achieving appropriate calibration decreases. Recent methodologies such as sensors and remote sensing offer interestingly high degrees of automation (in addition to miniaturization and simplification), but the difficulties posed by calibration make their most serious drawback relative to conventional manual methods. Automatic calibration (whether single or multipoint and for one or several analytes) applies to analytical processes, instruments and apparatuses, reference materials (whether certified or not), standards, and external or internal devices that can be used for calibration. CRMs are used to evaluate analytical processes, standards are used to calibrate both analytical processes and instruments, and external and internal devices are used to calibrate both instruments and apparatuses. Automatic calibration in on-line monitoring can be implemented in any easy, convenient way. There are 2 general approaches in this respect.

In one approach, calibration operations are performed with monitoring disruption. The completely continuous configuration to be used includes a switching valve to allow standards to be sequentially aspirated at appropriate time intervals. Standards can also be injected into the continuous analytical system by using a switching valve located before the injection valve. In the second approach, calibration operations are performed without monitoring disruption. The sample stream is continuously pumped into the system and the standards are sequentially injected into this stream, each yielding a peak the height of which is used for calibration (7). One of the most salient advantages of flow-through sensors is the way in which calibration can be implemented (e.g., by using a switching valve to sequentially introduce samples and washing-regenerating solutions and standards). On the other hand, in conventional probe-type sensors, calibration and regeneration or conditioning involve removal and dipping of the sensor probe, which calls for human participation in most instances.

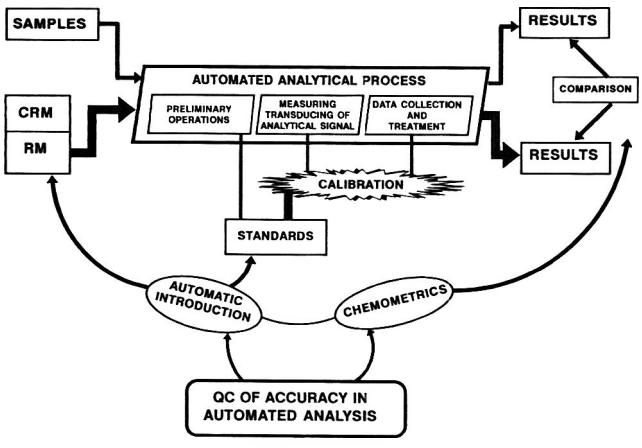

Figure 3. Main ways to control accuracy in automated analysis.

Enhancement of Analytical Quality Through Automation

Analytical quality is enhanced by reducing human participation in laboratory processes for 2 main reasons. The first is that analytical results are improved through reduction of human errors and simplication of SOPs (standard operational procedures). The second is that automation allows for increased control and convenient implementation of quality systems (quality assurance, quality control, and quality assessment). Computers and chemometrics enhance analytical quality through automation by fulfilling the following 3 related objectives: obtainment of more analytical information; optimization (in an automatic way) of analytical systems; and implementation of organization and management strategies, both in routine analytical laboratory work and in relation to quality systems.

Automation can efficiently help in enhancing both capital analytical properties (accuracy and representativeness) through such basic features as sensitivity, selectivity, and precision and analytical productivity, in terms of rapidity, costs, and human safety and comfort. Accuracy of the analytical results depends on the precision, sensitivity, and selectivity of the

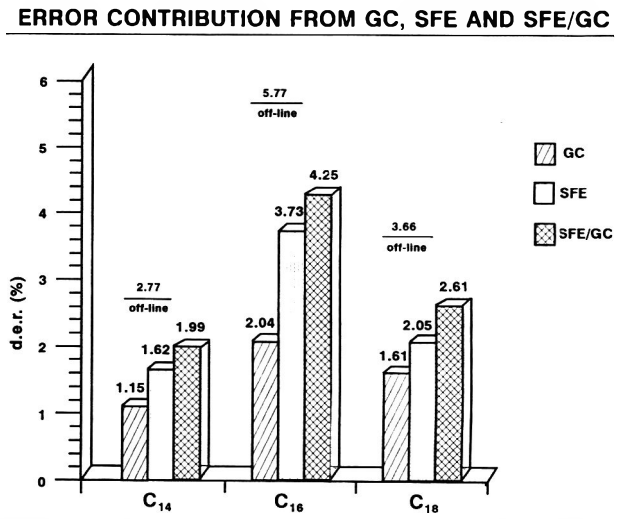

Figure 4. Systematic comparison of the errors obtained on the determination of some hydrocarbons in a polluted clay soil by using on-line (hyphenated) supercritical fluid extraction/gas chromatographic (SFE/GC) and off-line approaches.

analytical process concerned and can be indirectly enhanced by improving these basic analytical features. Such is the case with the use of an automatic sample/standard introduction system in electrothermal vaporization atomic absorption spectroscopy, where manual introduction affords a low precision relative to that obtained by using a sampler. Automation facilitates use of this technique for routine analyses.

It is a general principle that the higher the selectivity, the better the precision that can be achieved. By considering hyphenated techniques, the combination of discrimination of signals provided by instrumental techniques and separation of analytes allows errors to be reduced through decreased human participation. The errors obtained in the determination of some hydrocarbons (C_{14}, C_{16}, C_{18}, C_{10}) in a polluted clay soil (SRM from NIST) are shown in Figure 4. As can be seen, the errors yielded by a supercritical fluid extractor and a gas chromatograph coupled on-line are less than the sum of the errors obtained by applying the 2 techniques separately in an off-line approach.

The impact of automation on the productivity of an analytical laboratory is self-evident. The boosted sample throughput, reduced costs, and increased personnel safety and comfort are some clear assets of reduced human participation in the analytical process.

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AOAC SYMPOSIUM: AUTOMATED GOOD LABORATORY PRACTICE

Corruption at the Data Capture Stage and Good Laboratory Practices

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Possible sources of data corruption at the data capture stage include errors from the analogue input signal to be sampled, incorrect timing of the real-time sampling, loss of data on the data transmission path, and malfunctions of hardware and software components. Hardware and software measures to avoid such errors and provisions to adhere to good laboratory practice rules are discussed.

Reliable, high-quality analytical measurements are possible only if all sources of data corruption are carefully considered. This paper concentrates on the acquisition of analytical data with the aid of computer systems. The first part covers possible deficiencies and means to avoid them; the second part describes one of the data acquisition systems developed at the Max-Planck-Institut für Kohlenforschung.

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Possible Sources of Data Corruption

Special measures have to be taken if computerized data acquisition has to be done in "real-time," i.e., if the timing is controlled by an event, such as chromatographic elution, that takes place outside the computer, or if the signal to be measured is an analogue signal that has to be digitized before being processed by a computer program. The following sources of possible pitfalls are discussed in more detail: the analogue signal and its transmission path to the digitizer, the analogue-to-digital (a/d) converter (adc) system, timing of a/d sampling, and loss of data resulting from timing problems or malfunctions of hardware and software components.

Analogue signal.—Analogue signals are masked with high-frequency noise originating from the detector, the amplifying circuit, or the environment. Therefore, at least with low-level

signals, some kind of signal conditioning is usually required, either by smoothing the digitized signal or by applying low-pass filters to the analogue signal. In most systems, both methods are applied. It is good practice, however, to retain the untreated, digitized ("raw") data and to use smoothing only implicitly, e.g., within a software algorithm that searches for the beginning of peaks. This approach follows the important general principle of storing unmodified, raw data, not only for inspection purposes but also to allow for repeated analysis of the original data. High-frequency noise can also be suppressed by using integrating adcs with long integration times.

With low-level analogue signals, common-mode voltage can be a problem. Low-level signal measurement is differential, i.e., the data signal is the voltage difference between 2 input points. Common-mode voltage exists on each of the differential inputs and could be converted into normal-mode voltage by unbalanced impedances. This can be avoided by using twisted-pair cables with a common guard and proper grounding.

A/d converters and preamplifiers.—Most off-the-shelf a/d converters provide 12 to 16 bits of precision, which obviously is not sufficient to cover a large dynamic range required in signal sampling for methods such as gas chromatography (GC), where peaks range from microvolts to volts. This large dynamic range could be covered with logarithmic a/d converters, which require subsequent linearization. Linear a/d converters coupled with preamplifiers that are either programmable or work with auto-ranging hardware are applied more commonly. High-quality digitizing equipment is often too expensive to supply one for each instrument. It is more cost-effective for a large number of instruments to share in the hardware by applying multiplexer switching.

The use of different amplification ranges introduces an additional source of errors: Even if the adc itself provides good linearity, the amplifier hardware may switch into the wrong range or return an erroneous range code.

Outliers ("spikes") within the series of digitized data samples may result from badly adjusted hardware or electric disturbances from the environment. Before applying any smoothing, software should be used to check the data with an appropriate algorithm for detection and correction of spikes.

Precision of the adc normally is not very important for peak height and area measurement. If, however, a very small peak is located on top of a baseline offset, a situation that is often encountered with drifting baselines in temperature-controlled chromatography, the amplifier has to be switched into a less sensitive amplification range because of the absolute value of the offset-voltage. The resulting resolution may not be sufficient to detect the small peak. Therefore, a 16-bit-adc is useful, even if 8 bits of precision would be sufficient for the quantitative evaluation of a full scale peak.

Hardware calibration.—In laboratories where quality and repeatability of measurement are of major importance, periodic tests of the equipment should be performed. Calibration voltages can be fed into the analogue input channels to verify correct a/d conversion and/or software operation. Some commercial digitizing systems offer special calibration cards with precision voltage output for this purpose. Another good test is sampling a time-linear increasing voltage and checking a plot of the results. Feeding a reproducible artificial spectrum or chromatogram, e.g., via a peak generator, into the adc allows one to test the hardware as well as peak detection, and integration algorithms.

Data rates.—It is generally assumed that higher data rates (samples per second or per peak width) improve the accuracy in determining peak positions and areas. It has been shown (1, 2), however, that 12 samples over the ($\pm 3\sigma$–) width of a symmetrical Gaussian peak profile suffice to reduce the relative error for the area determination to <1%. The errors originating from baseline reconstruction and recognition of beginning and ending points of peaks are normally larger. High data rates not only consume compute time of the central processing unit (cpu) and disk storage but may result in unwanted "noise peaks" and in errors resulting from delayed recognition of the beginning of a peak.

Line frequency synchronization.—If low-level signals are measured with high sensitivity, some of the noise on the analogue signal originates from line frequency noise ("ripple"). If such a signal is digitized with a data rate r that is not an integer divisor of the power line frequency f_o, undesirable side effects may occur in form of difference frequencies: $\Delta f = f_o - k \times r$ (with k = 1, 2, ... , n) showing up in the digitized signal (Figure 1). In the case of very small differences, Δf (Figure 1c), a periodical baseline shift in the digitized data that is not present in the analogue chromatogram may be feigned. Such distortions can be avoided if data sampling is kept in phase with line frequency by synchronizing with hardware interrupts derived from the line frequency, or if an integrating adc is used with an integration time that is a multiple of the line frequency period, i.e., $\Delta t = k \times 20$ ms (3).

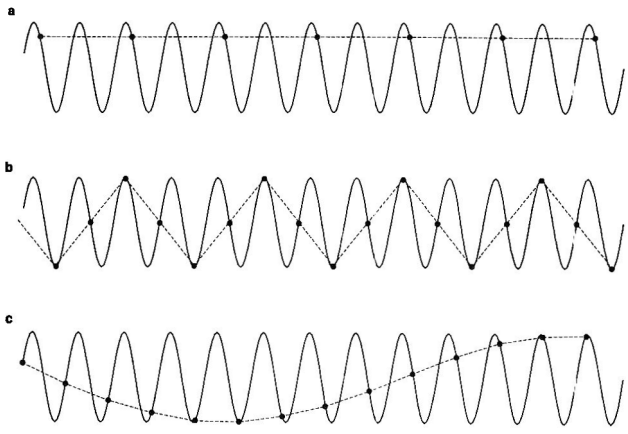

Figure 1. Analogue signal overlaid with line frequency noise (f_o) is sampled with data rate r in the following manner: (a) in phase with line frequency signal, no distortion of digitized signal ($k \times r = f_o$); (b) without line frequency synchronization, artificial signal Δf ($r = 4/3 \times f_o$; $\Delta f = r - f_o = 1/3 \times f_o$); (c) slightly out of phase with line frequency, artificial baseline drift.

Loss of data.—With a multichannel system, the total load on adc sampling and on required data throughput varies over time depending on the number of simultaneously active channels and on data rates. If no hardware or software precautions are provided, over-loading may occur, resulting in missed data because of data overrun or busy adc. Restrictions as to the maximum number of allowable channels and/or total data rate will cure the busy-adc problem. If the throughput is limited by the speed of processing or transfer of data to a host computer, temporary bottlenecks can be overcome by providing enough internal memory for data buffer space. It is common practice to process data by using buffer space: while a previously filled buffer (normally a fixed-length memory array) is processed, the next buffer is filled by new samples. (For multichannel systems it is advisable to have separate buffers for separate channels.) Data buffering is no cure, however, if real-time response is required, e.g., for instrumental control. If the data transmission path (e.g., the connection to a host computer or to external memory) is not available for a longer period of time, the users will not get any data output, and eventually even large "rings" of buffers will fill up, resulting in a forced stop of channels.

Data can be lost at any place on its way from the analogue input channel, through the data acquisition system, the network connection, and one or more host computers or servers, to the external storage medium. Software control of any loss of data is an absolute necessity. This can be achieved by setting software "flags" for specific data loss situations, e.g., if data cannot be acquired fast enough, within the data sampling module and by assigning sequential "block numbers" to each buffer being filled, preferably on a per-channel basis. Error messages can be sent if such an error flag or a missing block number is detected.

Malfunctions of hardware and software components.—Modern computer systems are much more reliable than systems 10 years ago. Mean-time-between-failures (MTBF) has improved tremendously, even for mechanical parts such as disk modules with MTBF-values of several years. Nevertheless, in addition to the operating system and network software, disk hardware is still the most important point of failure. Therefore, the adc-controlling data acquisition system should be kept as simple as possible: no moving mechanical parts, no complicated operating system (especially no multiuser system), and a simple but efficient data transmission software module that interconnects with some host computer. All dialogue interactions with the users should be with the host system only.

Hardware redundancy.—Except for the adc system and its controlling cpu, temporary malfunctions can be bridged by providing sufficient buffer memory. If, however, the resulting wait time, during which no interaction with newly acquired data is possible or no output of results is obtainable, is not tolerable for the users, redundant hardware components are needed (Figure 2).

The entire host system or any parts of it can be doubled. "Shadow-disks" with mirrored information or RAID (redundant array of inexpensive disks) technology can be used to prevent loss of data from disk crashes.

A more elegant way, which will bypass malfunctions of the hardware and avoid fatal operating system failures, is provided through a second host system: All data blocks from the data acquisition system that are sent to the host system are also sent to a "shadow host" system and are stored there in an identical file structure. (With network protocols that allow one to address a block of data to more than one receiver, it is not even necessary to send a block twice.) All software modules that are needed for the automatic part of the processing of data can be initiated in the shadow-host to stay in a sleep state until explicitly or automatically awakened after a failure of the primary host system. Some synchronization between the 2 systems is needed to keep the shadow system updated on the data already processed.

With today's hardware prices, a shadow-system should not be too expensive for situations where extreme reliability is required. In contrast, a redundant data acquisition system would be more expensive because of the cost of a/d hardware and of additional analogue cabling and the proper grounding of parallel systems.

Good laboratory practice (GLP) requirements.—GLP regulations require measures against artificial modifications of raw data files as well as bookkeeping information for keeping track of the history of data. To comply with these regulations, the data acquisition system should supply date and time stamps as integral parts of the data files. Furthermore, raw data files should be secured against subsequent modifications by disallowing any write-access to those files.

Implementation of a Data Acquisition System for Chromatography

In the Max-Planck-Institut für Kohlenforschung, the first systems for the real-time acquisition of data from analytical instruments were developed between 1968 and 1970 (4). A multiuser, time-sharing DEC10 computer was connected on-

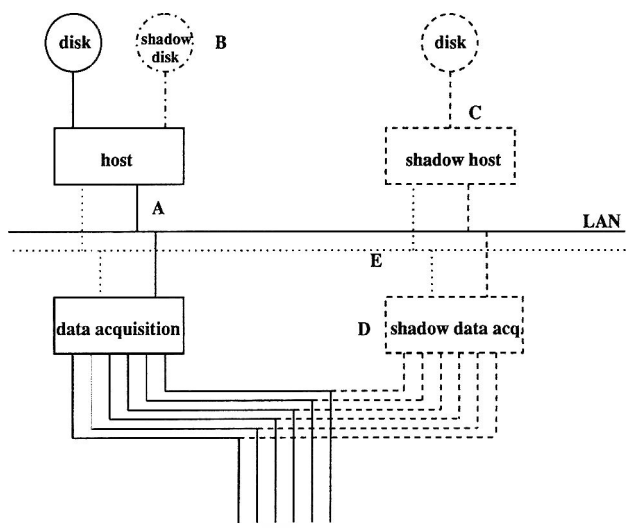

Figure 2. Different levels of hardware redundancy: (A) data acquisition system connected to host computer via LAN, (B) shadow disk or RAID, (C) shadow host, (D) shadow data acquisition system, and (E) redundant network hardware.

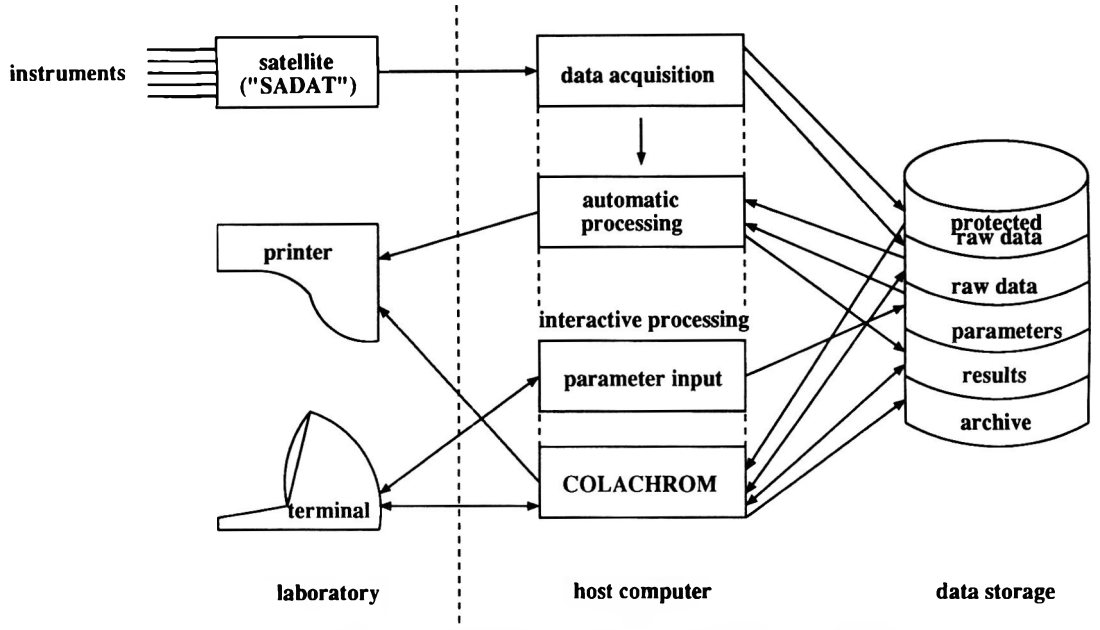

Figure 3. Data flow in the chromatography computer system. Signals from the instruments are sampled by the SADAT satellite and transferred to a host computer for storage and automatic processing. The COLACHROM software allows for subsequent interactive processing.

line to chromatographs, optical spectrometers, nuclear magnetic resonance instruments, and mass spectrometers. Its operating system was modified to allow for these real-time applications. That system was supplemented over time with several instruments controlled by minicomputers but stayed in operation until 1981, when it was replaced by VAX computers (5). At that time, however, the data systems for the on-line applications were redesigned, which resulted in a separation of the real-time tasks from data storage and data interpretation tasks. For the real-time tasks, different dedicated LSI11 microcomputer satellites were implemented with hardware and software specialized for GC/mass spectrometry (MS) with fast-scan low resolution mass spectrometers (6), GC/MS with a quadrupole instrument (7), and chromatographs and other slow-scanning analytical instruments (8, 9). All of these satellite computers have no moving mechanical parts (such as disks) or general-purpose operating systems; instead, they have software tailored to the application and a network connection to host computers. These satellites are still in operation, whereas the VAX computers have gone through several generations.

The CHROMDAT data acquisition system for chromatography consists of different tasks (Figure 3), one of which, the real-time servicing of instruments ("SADAT"), is assigned to a microcomputer satellite.

Five satellite systems for chromatography are located in different laboratories and are connected via an Ethernet local area network to VAX/VMS host computers, where the data are stored and finally processed. (Copies of the described system are in operation in several laboratories outside of the institute.)

The satellite's software, called SADAT, is down-loaded from the host computer via a load-module residing in EPROM memory of the LSI11 microcomputer and is automatically restarted by a power-up of the processor. In the case of a host restart, SADAT automatically logs into the host and starts the READER process there, whose task is to receive all data from and to communicate with the satellite. If the host goes down, SADAT temporarily buffers all incoming data in internal memory until the host is up again. Four megabytes of buffer memory is sufficient to store about 100 chromatograms, which is approximately the average production of one work day.

Other functions of SADAT include the following: servicing of start/stop requests for instruments (push-button requests as well as programmed requests from host); sampling of up to 40 analogue input channels with data rates that are either selectable, synchronized by line frequency, and defined by $f = f_o / 2^n$, with f_o = line frequency (e.g., 50 Hz) and n = 1, 2, ..., 15, or are triggered by switching of an external relay; sampling of up to 8 digital channels with serial interfaces (RS232C, IEC); transfer of data in blocks of 128 samples to a host computer via an Ethernet-interface, or an R232C serial line; recognition of auto sampler rack/vial codes; instrument control via relay switching; recording of bookkeeping information such as date and time of channel starts, data rate, run time, and accumulative statistics; and periodical update of channel parameters in memory-resident tables of the host computer.

A/d hardware in the form of different subsystems ("RTP"-hardware manufactured by Computer Products Inc, Florida) is supported with autogain or programmed gain ranging from about 1 μV to 10 V dynamic range.

Reliability.—Since 1981, the SADAT program underwent only one major modification, when support for an Ethernet interface was included in 1985. The satellites operate extremely reliably. The main points of failures are outages of the public mains. Very few amplifier cards of the RTP hardware had to be replaced over the years. Because of the high reliability, no hardware redundancy is deemed necessary. The most unstable com-

ponent in the overall system was the host computer with its multiuser operating system (VMS) and its disk storage. No shadow-host was installed, however, because the satellites can bridge many hours of down-time by buffering data in internal memory.

Related software.—At the host computer, data evaluation according to preselected parameters is automatically started as soon as data acquisition for a channel is finished. The result — in essence a list of peaks — is written into a "report file" and stored together with the file of raw data into the disk directory of the associated user. The user can pick up those files for interactive processing with COLACHROM (for chromatography) or PIPSI (for optical spectroscopy).

GLP features.—The date and time stamp provided for each start of data acquisition is stored within the file of raw data, together with the analogue channel, the rack number and vial position of the autosampler (if available), the data rate and total run time, and a flag to characterize the reason for the data acquisition being finished (push-button, time-out of preset run time) or any error condition.

If desired by laboratory management, the host system, via logical variables set by the system manager, can be tailored to write a copy of the original file of raw data into a special disk directory that is protected against any user modifications. When data are archived (ARCHIVE command in COLACHROM), this original file is copied together with the possibly modified file of raw data and the report file generated by the user into an archive directory, which is periodically transferred onto some long-term storage medium.

The report file holds all parameters used for processing, such as baseline reconstruction and peak detection, as well as date and time of the last modification and the name of the last modifying user. If command procedures have been applied for semi-automatic processing and report generation, COLACHROM also stores the names of the last 10 procedures used. Information such as sample identification, customer identification, and instrumental conditions are introduced interactively by the user either before starting the acquisition or in the course of processing the data.

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